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PRINCIPAL INVESTIGATOR: Georg F. Weber, M.D., Ph.D.

CONTRACTING ORGANIZATION: New England Medical Center
Boston, Massachusetts 02111

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Georg F. Weber, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
New England Medical Center
Boston, Massachusetts 02111

E-Mail: gweber@lifespan.org

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13. ABSTRACT ***(Maximum 200 Words)***

Cancer is characterized by dysregulated growth control, overcoming of cellular aging, and metastasis formation. The latter distinguishes malignant tumors from benign tumors and is mediated by groups of molecules called homing receptors, cytokines, and proteinases. Expression of these molecules on tumor cells determines when and where particular types of cancer spread.

The physiologic role of the relevant receptors, cytokines, and proteases in the healthy, cancer-free organism has been incompletely understood. We have studied a homing receptor, known as CD44, and its ligand, the cytokine osteopontin. In host defense, CD44 and osteopontin play key roles in mediating delayed types of immune response that are important in tuberculosis, organ transplantation, and many forms of vaccination. Macrophages are the cells that mainly determine whether an immune reaction will have delayed (cell-mediated) or acute (antibody-mediated) characteristics, and osteopontin and CD44 direct macrophages to the former. The engagement of CD44 by osteopontin also induces macrophage migration, a mechanism that metastatic tumors may utilize in the process of dissemination. We have found that other gene products that contribute to dissemination of cancerous cells similarly contribute to host defenses, and we conclude that metastasis genes are developmentally non-essential genes which physiologically mediate stress responses, inflammation, wound healing, and blood vessel formation.

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INTRODUCTION

Malignancies display invasive behavior that is conveyed by homing receptors (and associated signal transduction molecules), their ligands (mostly cytokines), and secreted extracellular-matrix-degrading proteases. The topology of metastasis formation is determined by the specific identity of the homing receptors expressed on the tumor cell surface and their ligands (Weber/Ashkar 2000). Although the mechanisms regulating such invasive tumor cell migration and subsequent implantation are incompletely understood, there is abundant evidence that the cytokine osteopontin can mediate the dissemination of cancer cells. It constitutes the major phosphoprotein secreted by transformed cells (Senger et al. 1980; Senger et al. 1983; Senger et al. 1989; Chambers et al. 1992) and its levels correlate with metastatic potential (Craig et al. 1990; Senger et al. 1989). Transfection of tumor cell lines with osteopontin increases their malignant phenotype (Denhardt/Guo 1993) while transfection with osteopontin antisense oligonucleotides yields populations with reduced malignant potential (Behrend et al. 1994; Gardner et al. 1994). Induction of osteopontin has been shown to be sufficient for metastasis formation by mammary epithelial cells (Barraclough et al. 1998; El-Tanani et al. 2001).

Splice variants of the homing receptor CD44 mediate metastasis formation by various tumors. Expression of the cytokine osteopontin has been associated with the malignant potential of tumor cells. We have identified a novel receptor/ligand interaction between CD44 and osteopontin that mediates cell attachment or migration. Both molecules are subject to multiple posttranscriptional and posttranslational modifications and our data suggest that these alterations modulate binding. Organ-preference in metastasis formation may be based on specific recognition via homing receptors and modifications in CD44/osteopontin interaction may provide a potential molecular explanation for some aspects of it. To assess why breast cancer metastasizes predominantly into bone, we are investigating modifications in both proteins that allow interaction.

BODY

Specific Aim 1: Identification of the posttranslational modifications in both proteins which are permissive for interaction.

Task 1: Purify CD44 from breast cancer cells and analyze splice variants as well as posttranslational modifications.

A splice variant of CD44 containing the variant exon 6 is frequently expressed on malignant cells, but not in the healthy adult organism. In our original identification of the CD44/osteopontin interaction (Weber et al. 1996) we had not dissected whether the osteopontin binding site on CD44 is located on the standard form or on any of the variant exons. We realized that the standard form does not bind this ligand and communicated the result in a letter to the editor (Science 1998; 282:243). This observation was confirmed and extended by other investigators (Katagiri et al. 1999).

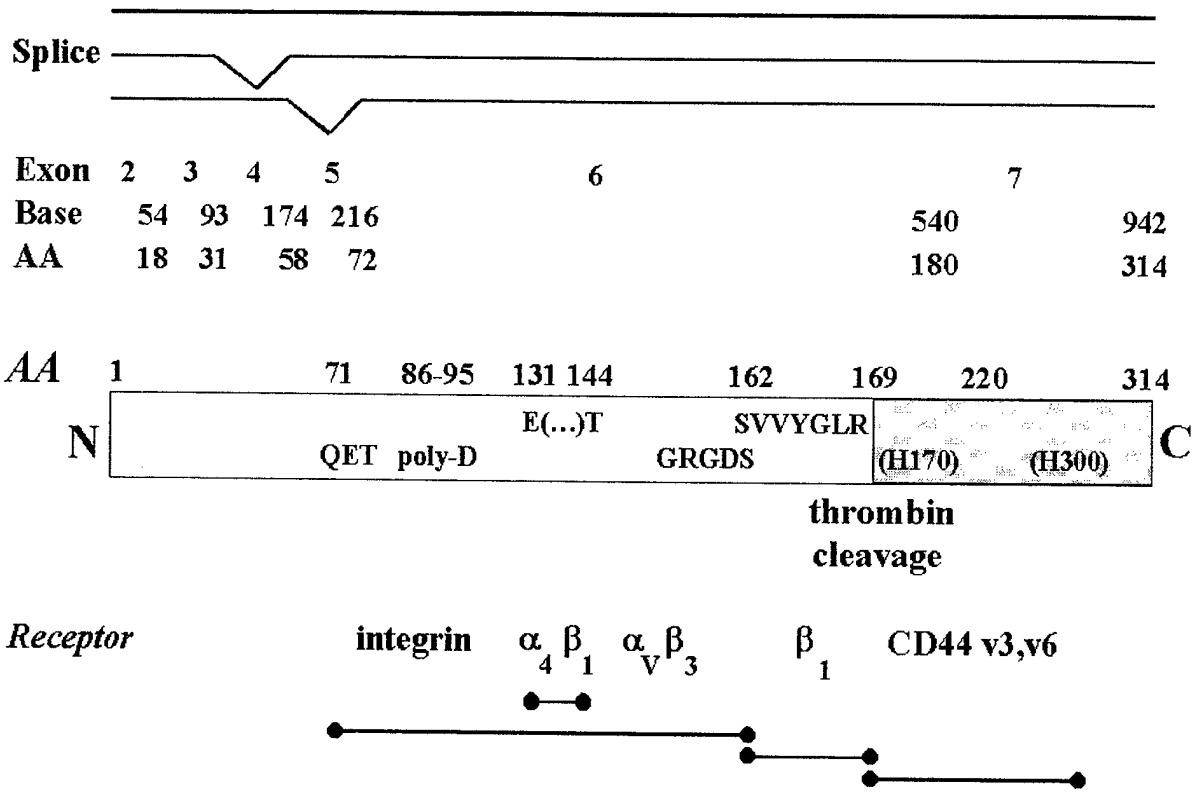
We had previously performed structure activity analyses of osteopontin and its receptors in macrophage migration and activation as a model for metastatic spread and found that an interaction between the C-terminal domain of osteopontin and the receptor CD44 induces macrophage chemotaxis, while engagement of integrin receptors by a non-overlapping N-terminal osteopontin domain induces cell spreading. Serine phosphorylation of the osteopontin molecule on specific sites is required for functional interaction with integrin but not CD44 receptors. A manuscript detailing the structure/activity analysis of osteopontin has recently been accepted for publication in the Journal of Leukocyte Biology (Weber et al. 2002). The associated signal transduction pathways are still subject to further study (compare Task 7).

Task 2/3: Purify and compare the two forms of osteopontin according to structure and function with regard to breast cancer cell migration.

Biological functions of metastasis associated gene products are extensively regulated at the posttranscriptional and posttranslational levels (Weber/Ashkar 2000). Consistently, osteopontin from various cellular sources may have diverse structural characteristics (Kon et al. 2000). The main physiologic source of osteopontin is $CD4^+$ T-

cells. Macrophages may also produce osteopontin after stimulation with lipopolysaccharide but with distinguishable biological consequences. The macrophage-generated osteopontin is structurally different from the T-cell derived molecule, possibly due to loss of part of its sequence by alternative splicing (S. Ashkar and G.F. Weber, unpublished observations). Studies in osteopontin$^{-/-}$ gene targeted mice (Crawford et al. 1998) have suggested the existence of structural and functional differences between tumor-derived osteopontin and the osteopontin forms that are relevant for host defenses. Evidence suggests that tumor-derived osteopontin is unique (i.e. structurally different from osteopontin derived from non-transformed cells) and lacks important domains (Figure 1). As a case in point, an osteosarcoma secreted a smaller form of osteopontin than the predominant product secreted by non-transformed bone cells (Kasugai et al. 1991). Malignant cells often secrete hypophosphorylated osteopontin variants (Shanmugam et al. 1997) or a splice variant that has a deletion in its N-terminal portion (Kiefer et al. 1989) and this molecule may contribute to metastatic spread (Weber et al. 1997) by inducing cell migration. Concomitantly, hypophosphorylated or alternatively spliced osteopontin could ligate CD44 on macrophages leading to chemotaxis (Weber et al. 1996) and suppression of IL-10, but would possibly engage its integrin receptors less efficiently, so that substantial amounts of IL-12 would not be secreted (Ashkar et al. 2000). Due to the inability of tumor-derived osteopontin to associate with the extracellular matrix (Rittling et al. 2001) its cytokine functions (dependent on its presentation in soluble form (Adler et al. 2001)) may be particularly prominent. Thus, expression of structurally altered, spliced or hypophosphorylated osteopontin by cancer cells may, among other functions, represent a mechanism of immune evasion.

Figure 1: Structural characteristics of the osteopontin gene product. *Top: The gene has 6 translated exons. Sequences for splice variants of exons 4 and 5 are deposited in Genbank. Middle: The protein contains two main domains, a N-terminal fragment contains the integrin binding sites, while the CD44 binding site lies on the C-terminal domain. Bottom: The integrin binding site covers the sequence GRGDS. The smallest integrin $\alpha_V\beta_3$ binding peptide identified by us starts at AA71. Binding to β_1-containing integrins occurs through the non-canonical sequence SVVYGLR, unless the β_1 chain is paired with α_4, in which case the binding site ranges from AA131 to AA144. We have found the CD44v6 binding site to cover the region from AA169 to AA220. Heparin-bridges between osteopontin and CD44v3 may be formed via the heparin binding sites on AA170 and 300. The scheme is not drawn to scale.*



Several reports (Kon et al. 2000; Kasugai et al. 1991; Shanmugam et al. 1997; Kiefer et al. 1989; Weber et al. 1997), some of them recent (Crawford et al. 1998), have shed light on the structural and functional differences between tumor secreted osteopontin and host osteopontin. Host osteopontin may protect from tumors while osteopontin produced by cancer cells confers metastatic potential (Crawford et al. 1998; Denhardt/Chambers 1994; Feng et al. 1995). We had reason to hypothesize that the structural variations in osteopontin from various sources might originate not only in posttranslational modifications, but also on the level of RNA editing. We have therefore analyzed the osteopontin messages in various normal and transformed breast epithelial cell lines by RT-PCR, cloning, and sequence determination (Figure 2). We also tested transformed epithelial and mesenchymal cells (MDA-MD-435, 21PT, 21NT breast cancers, LnCAP prostate cancer, SAOS-2 osteosarcoma). In these experiments, cells are starved (serum-free medium), treated with PMA (a potent inducer of osteopontin expression in many cells) or cultured in complete (serum-containing) medium. Osteopontin RNA is extracted, amplified by RT-PCR, cloned and sequenced. We have

found the expression of two forms in malignant, but not benign cells. Interestingly, normal breast epithelial cells (generously provided by Dr. Vimla Band; Liu et al. 1996; Ratsch et al. 2001) express low or moderate amounts of standard osteopontin, but no smaller transcript. After immortalization with the human papillomavirus oncogene E6, however, expression of two osteopontin bands is observed. The two bands were identified as the wild-type osteopontin and a splice variant missing the third translated exon (exon 4, because exon 1 is untranslated (Behrend et al. 1993)). The identified transcripts correspond to the sequences of "osteopontin-a" and "osteopontin-b", which have been deposited in GenBank (Accession numbers D28759 and D28760) but have not been thoroughly studied. Published reports on the expression or function of splice variants are limited to the observation of three types of osteopontin RNA messages in human gliomas (Saitoh et al. 1995) and to the existence of a splice variant that has a deletion in its N-terminal portion (Kiefer et al. 1989). It may be important to note that it is not unexpected for tumors to express multiple variant transcripts of metastasis-associated genes. Similar observations have been made for the homing receptor CD44. Tumors may express up to nine distinct spliced forms of this gene (Matsumura/Tarin 1992). Only certain CD44 splice variants support metastasis (Gunthert et al. 1991), while the standard form acts as an inhibitor of dissemination (Tanabe et al. 1995). It can be hypothesized that similar relationships may apply for standard osteopontin and its splice variant. This might account for the apparently conflicting observations that osteopontin produced by cancer cells (the unique form being osteopontin-b) supports their dissemination, while host osteopontin (osteopontin-a) may contribute to anti-cancer immune surveillance.

Because osteopontin is not spliced in benign tumor cells or in T-lymphocytes the domain encoded by exon 4 may be essential for host defense. The splicing deletes a polypeptide that does not have any known functional motifs and interferes only minimally with the previously defined binding region for integrin $\alpha_V\beta_3$ (Figure 2). A homology search in the protein database (pdb) has yielded only rudimentary homology with other polypeptides, whose origins range from viral to xenopus-derived. No specific function can be inferred from the homology search.

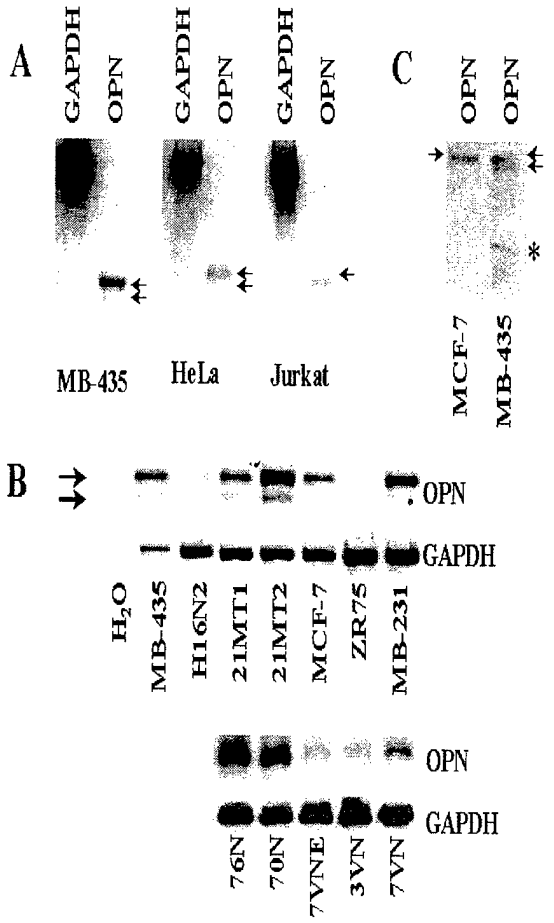


Figure 2: Expression of osteopontin splice variants in multiple tumor cell lines. *A,B) RNA was extracted from various cell lines, was reverse transcribed, and used as template in PCR reactions. Primers for osteopontin (OPN) amplified a 616 bp segment from the 5' end of the transcript. Alongside every other cell line, MDA-MB-435 cDNA was amplified to mark the two osteopontin bands (not shown). Resulting double bands (MDA-MB-435 cells, HeLa cells, 21MT cells, not shown: Saos-2 cells) were cloned and sequenced. No template (not shown) and GAPDH served as controls. A) In malignant breast cancer (MDA-MB-435) and lymphoma (HeLa), two osteopontin bands are amplified. In T-cells (Jurkat) one band is obtained. B) (top panel) Osteopontin expression in breast tumor cell lines. Two bands are seen in the malignant cells (MDA-MB-435, 21MT1, 21MT2, MDA-MB-231). In benign cells (H16N2, MCF-7, ZR-75) one or no band is obtained. (bottom panel) Normal breast epithelial cells express low or moderate amounts of standard osteopontin (76N, 70N, 7VNE, 3VN, 7VN). In contrast, breast epithelial cells immortalized with the HPV oncogene E6 (81E6, M2E6E7, 16E6P) express two transcripts of osteopontin (not shown). C) The number of transcripts detected by RT-PCR corresponds to the number of protein bands on Western blots from cell lysates. The * indicates a likely cleavage product that is very commonly observed on Western blots for osteopontin.*

We have found an osteopontin splice variant to be expressed in breast cancer cells, but not in benign breast tumor cell lines. Even though no unique structural characteristics have been identified for the spliced exon, an existing literature has described structural (migration on SDS-PAGE) and functional (metastasis versus host defense) differences between tumor-derived and host-derived osteopontin that may be explained by differential splicing. A recent publication (Rittling et al. 2001) has reported on the inability of tumor secreted osteopontin to mediate cell adhesion to the extracellular matrix, providing a possible criterion for differentiating standard and spliced osteopontin.

We will follow up on these preliminary observations. We are also setting up experiments to immunoprecipitate osteopontin from breast cancer cells (MDA-MB-435 and MDA-MB-231, which secrete osteopontin constitutively) and from bone-derived cells (the target for breast cancer cell invasion) cultured in serum-free medium. We will attempt to analyze the partially purified protein by mass spectrometry for

phosphorylation and glycosylation. The Dana-Farber Cancer Institute has a molecular biology core facility that performs similar analyses routinely and has performed studies of posttranslational modifications for us previously. It should be noted, however, that the complexity of the starting material often allows only partial characterization of the protein.

Task 4: Define binding sites on CD44 and osteopontin and the consequences of their interaction.

Cancers of particular tissue origin show consistent preference for specific target organs to spread to. This topology of metastasis formation is mediated by the potpourri of homing receptors on the tumor cell surface and their ligands and is widely believed to have its physiologic correlate in morphogenesis during embryonic development. Unexpectedly, knockout mice in which individual genes known to participate in tumor spread were disrupted turned out to be fertile and developmentally normal. The defects observed in the relevant gene targeted mice are impairments of various features of stress responses. Metastasis-associated gene products have several characteristics in common. They comprise a set of developmentally non-essential genes, which physiologically mediate stress responses, including inflammation, wound healing, and neovascularization. Recognition of topology is encoded in the surface molecules of immune cells and organ preference by cancer may be derived from a process which immune cells use to target their responses. Metastasis-associated gene products therefore constitute a unique and essential group of cancer-related biomolecules. We conclude that metastasis formation is a process that mimics macrophage behavior (Weber/Ashkar 2000a,b). We believe that this insight constitutes a major paradigm shift for two reasons 1) the classical cancer related genes (oncogenes, tumor suppressor genes, telomerase, and DNA repair genes) do not account for metastasis formation, 2) currently, most metastasis researchers favor the assumption that metastasis formation copies mechanisms of organ development; this notion, however, is clearly refuted by the absence of developmental abnormalities in the relevant knockout mice.

We attempted to define the consequences of interactions between CD44 and osteopontin in Boyden chambers, where the upper surface of the trans-membrane was

coated with matrigel to measure cell invasion. Those experiments were unsuccessful due to the interference of laminin (the major constituent of matrigel) with the effects of osteopontin on cell motility (similar, yet unpublished observations were made with regard to the laminin effect on osteopontin-mediated T-lymphocyte co-stimulation). The identification of this laminin effect may potentially lead to treatment options for metastatic tumors.

Mice deficient in osteopontin gene expression fail to develop delayed type hypersensitivity responses or Th1-driven autoimmunity after viral or parasitic infection. These defective immune responses are associated with diminished production of the stimulatory cytokine IL-12 and excessive production of the inhibitory cytokine IL-10. A phosphorylation-dependent interaction between the N-terminal portion of osteopontin and its integrin receptor on macrophages leads to IL-12 expression, while a phosphorylation-independent interaction of osteopontin with CD44 inhibits IL-10 expression. (Ashkar et al. 2000).

Based on the immunomodulatory effect of osteopontin, we have designed candidate peptide drugs that selectively interfere with receptor binding to either CD44 or integrin $\alpha_V\beta_3$ (patent pending; see appendix).

Specific Aim 2: Investigation into the roles of CD44 and osteopontin in metastasis formation.

Task 5: Transfect benign cells with the cancer derived CD44 splice variant or osteopontin and test the malignant potential. Transfect malignant cells with antisense constructs and test amelioration of malignancy.

We have back-crossed CD44 knockout mice with mice bearing mutant p53 or APC tumor suppressor genes to assess the influence of CD44 on tumorigenicity. Mice with point mutations in tumor suppressor genes, $APC^{+/min}$ bred on C57Bl/6 background or $trp53^{+/tm1}$ on C57Bl/6 background, were obtained from Jackson Laboratory. Either $APC^{+/min}$ mice or $trp53^{+/tm1}$ mice were mated with $CD44^{-/-}$ mice that had been back-crossed from 129 to C57BL/6 for 4 generations (Su et al. 1992). The genotypes were assessed using PCR

from genomic DNA (Schmits et al. 1997; Jacks et al. 1994; Su et al. 1992) and CD44 expression was confirmed by flow cytometry from blood samples using the pan-CD44 antibody IM7 (Pharmingen). Siblings were housed in groups of 1-4 per cage at the Redstone Animal Facility (DFCI) in alternate 12-hr light and dark cycles. A diet of pelleted chow (Agway, Prolab 3000) and bottled water was administered ad libitum and room temperature was kept at 25°C. The colony was frequently tested for endoparasitic and ectoparasitic infections, as well as for bacterial and viral infections by the Charles River Labs (Wilmington MA). No infection was detected during the course of this study. Permission to exceed a tumor diameter of 2 cm was granted by the institutional animal care and use committee and the mice were frequently seen by a veterinarian.

Because aberrant expression of CD44 splice variants may confer a malignant phenotype to tumor cells, we asked whether the targeted deletion of the CD44 gene was sufficient to suppress the dissemination of solid tumors. Osteosarcomas developed mostly on the lower back. One $trp53^{+/tm1}CD44^{-/-}$ mouse had an osteosarcoma of the skull. Metastases were detected in the lungs and livers from $trp53^{+/tm1}$ mice with osteosarcoma. Step sections from livers and lungs identified 28 metastases in 6 $CD44^{+/+}$ mice and 1 metastasis in 4 $CD44^{-/-}$ mice. One $CD44^{+/+}$ mouse also displayed a macroscopically visible metastasis in the spleen. All 6 $CD44^{+/+}$ mice had multiple osteosarcoma metastases while in 4 $CD44^{-/-}$ mice only one individual lung metastasis was detected. Consistently, CD44 expression was prominent in the osteosarcomas of $CD44^{+/+}$ mice.

The absence of CD44 does not alter the phenotype of benign tumors. The intestinal polyps caused by mutation in the APC gene grow non-invasively but express various splice variants of CD44. This occurs at the earliest stages of transformation diagnosed as aberrant crypt foci with dysplasia. Histology was performed on the largest intestinal polyps from each $APC^{+/min}$ mouse to assess malignancy. Consistent with previous reports, these tumors are non-invasive as judged by intact basement membranes in all cases. No metastases were observed in other organs (3 histologic sections per organ). These results were not affected by the presence or absence of CD44. Histologic findings included ectopic hepatic hematopoiesis and bone marrow siderosis in several mice, which are likely attributable to blood loss through the intestinal polyps. In all tumors studied, the absence of CD44 gene products did not affect tumor incidence or survival. Therefore, CD44 gene products are not

essential for tumor incidence and growth, but are important in regulating metastasis formation. The manuscript has been published in Cancer Research.

Task 6: Inject breast cancer cells and test the preferred site of metastasis formation.

We have transfected cells that do not express endogenous CD44 (the Balb/c derived leukemia cell line S49 as well as osteosarcoma cells derived from a mouse with mutant p53 and gene knockout of CD44 that had been bred and raised in our laboratory) with a retroviral vector containing GFP and, in a separate open reading frame, either no gene, CD44v4-10, CD44v7-10, or CD44v8-10. We intend to use these cell lines for in vitro and in vivo experiments.

Task 7: Assess the potential contribution of osteopontin-mediated anti-apoptotic effects on tumor growth and spread.

We have characterized osteopontin-mediated homing as a two-step process mediated by the two main receptors, CD44 and integrin $\alpha_v\beta_3$ (Weber et al. 2002). In an effort to better understand the mechanisms by which osteopontin mediates cell invasion and survival through its receptors CD44 and integrin $\alpha_v\beta_3$, we have investigated signal transduction events induced by the cytokine after ligation of each receptor. An interaction between the C-terminal domain of osteopontin and the receptor CD44 induces macrophage chemotaxis via G-protein signaling, while engagement of integrin receptors by a non-overlapping N-terminal osteopontin domain induces cell spreading, mediated by PKC, and macrophage activation involving phosphatidylinositol as a secondary signal transduction component. Preliminary studies indicate that signaling mediated by phosphatidylinositol 3-kinase also contributes to the osteopontin-induced anti-apoptotic functions. Two manuscripts are in preparation.

In sum, we have analyzed the metastasis genes CD44 and osteopontin. To better understand their role in pathophysiology, we have investigated their physiologic roles in host defense and stress responses. Not all studies were focused on breast cancer. This was in part due to circumstance. The Department of Cancer Immunology and AIDS at the Dana-Farber Cancer Institute was not set up for breast cancer research. In some studies,

the available murine model systems to answer important questions were related to cancer, but not specifically to breast cancer. Finally, we believe that an understanding of metastasis formation requires the investigation of the physiologic roles of the relevant genes. This research inevitably led us back to immunology. Nevertheless, we believe that our work over the past four years has made substantial progress in defining metastasis genes as a unique group of cancer-related genes beside oncogenes and tumor suppressor genes. We have further define the regulation of metastasis genes to occur on the levels of aberrant expression or splicing and of post-translational modifications. We hope that these insights will have a substantial influence on future breast cancer research. Without the support from the USAMRMC these studies would not have been possible. With the continued generous support from the U.S. Army breast cancer program (BC011270), our laboratory is now investigating the molecular connections between oncogenes and metastasis genes.

KEY RESEARCH ACCOMPLISHMENTS

* Identification of CD44 as a metastasis gene that does not affect tumor incidence or growth

* Specification of the physiologic roles of osteopontin and of the division of labor between its two main groups of receptors, CD44 and integrins.

* Characterization of signal transduction events associated with ligation of CD44 and integrin $\alpha_V\beta_3$ by osteopontin

* Observation of an osteopontin splice variant selectively expressed in malignant tumors

* Identification of malignant tumor cells as a source of osteopontin splice variants

* Characterization of metastasis genes as stress response genes

REPORTABLE OUTCOMES

Ashkar S, **Weber GF**, Panoutsakopoulou V, Sanchirico ME, Janssen M, Zawaideh S, Rittling S, Denhardt DT, Glimcher MJ, Cantor H. Eta-1 (Osteopontin): An early component of type 1 (cell-mediated) immunity. Science 2000; 287: 860-864.

Weber GF, Ashkar S. Stress response genes - the genes that make cancer metastasize. Journal of Molecular Medicine 2000; 78: 404-408.

Weber GF, Ashkar S. Molecular mechanisms of tumor dissemination in primary and metastatic brain cancers. Brain Research Bulletin 2000; 53: 421-424.

Adler B, Ashkar S, Cantor H, **Weber GF**. Costimulation through adhesion molecules determines the response to ligation of the T-cell antigen receptor. Cellular Immunology 2001; 210: 30-40.

Weber GF. The metastasis gene osteopontin: A candidate target for cancer therapy. Biochimica et Biophysica Acta 2001; 1552:61-85. Reviews on Cancer online: http://www.elsevier.nl/gej-ng/10/38/20/48/35/00087506.pdf

Weber GF, Bronson R, Ilagan J, Cantor H, Schmitz R, Mak TW. Absence of CD44 prevents sarcoma metastasis. Cancer Research 2002; 62: 2281-2286.

Weber GF, Zawaideh S, Kumar VA, Glimcher MJ, Cantor H, Ashkar S. 2002. Phosphorylation-dependent interaction of osteopontin with its receptors regulates macrophage migration and activation. The Journal of Leukocyte Biology, in press.

He B, **Weber GF**. 2002. A non-canonical pathway to NF-κB activation. Manuscript submitted for publication.

Ashkar S, Cantor H, Glimcher MJ, **Weber GF** (inventors). Methods and compositions for modulating immune responses (U.S. patent 60/129,772 pending)

CONCLUSIONS

Cancer is characterized by dysregulated growth control, immortalization, and metastasis formation. The latter distinguishes malignant tumors from benign tumors and is mediated by groups of molecules that include homing receptors, cytokines, and proteinases. Expression of these molecules on tumor cells determines when and where particular types of cancer spread. The physiologic role of the relevant receptors, cytokines, and proteases in the healthy, cancer-free organism has been incompletely understood. We have studied the homing receptor CD44 and its ligand, the cytokine osteopontin. In host defense, CD44 and osteopontin play key roles in mediating delayed types of immune response that are important in tuberculosis, organ transplantation, and many forms of vaccination. Macrophages are the cells that mainly determine whether an immune reaction will have delayed (cell-mediated) or acute (antibody-mediated) characteristics, and osteopontin and CD44 direct macrophages to the former. The engagement of CD44 by osteopontin also induces macrophage migration, a mechanism that metastatic tumors utilize in the process of dissemination. We have developed a two-step model of breast cancer invasion based on the interactions of osteopontin and its receptors, integrins and CD44. We have found that other gene products that contribute to dissemination of cancerous cells similarly contribute to host defenses, and we conclude that metastasis genes are developmentally non-essential genes which physiologically mediate stress responses, inflammation, wound healing, and blood vessel formation.

REFERENCES

Adler B, Ashkar S, Cantor H, Weber GF. Costimulation by extracellular matrix proteins determines the response to TCR ligation. Cell Immunol 2001;210:30-40.

Ashkar S, Weber GF, Panoutsakopoulou V, Sanchirico ME, Janssen M, Zawaideh S, Rittling S, Denhardt DT, Glimcher MJ, Cantor H. The cytokine Eta-1/Opn is an essential molecular bridge to type 1 (cell-mediated) immunity. Science 2000;287:860-864.

Barraclough R, Chen HJ, Davies BR, Davies MP, Ke Y, Lloyd BH, Oates A, Rudland PS. Use of DNA transfer in the induction of metastasis in experimental mammary systems. Biochem Soc Symp 1998;63:273-294.

Behrend EI, Chambers AF, Wilson SM, Denhardt DT. Comparative analysis of two alternative first exons reported for the mouse osteopontin gene. J Biol Chem 1993;268:11172-11175.

Behrend EI, Craig AM, Wilson SM, Denhardt DT, and Chambers AF. Reduced malignancy of ras-transformed NIH 3T3 cells expressing antisense osteopontin RNA. Cancer Res 1994;54:832-837.

Chambers AF, Behrend EI, Wilson SM, and Denhardt DT. Induction of expression of osteopontin (OPN; secreted phosphoprotein) in metastatic, ras-transformed NIH 3T3 cells. Anticancer Res. 1992;12:43-48.

Craig AM, Bowden GT, Chambers AF, Spearman MA, Greenberg AH, Wright JA, McLeod M, and Denhardt DT. Secreted phosphoprotein mRNA is induced during multi-stage carcinogenesis in mouse skin and correlates with the metastatic potential of murine fibroblasts. Int J Cancer 1990;46:133-137.

H.C. Crawford, L.M. Matrisian, L. Liaw, Distinct roles of osteopontin in host defense activity and tumor survival during squamous cell carcinoma progression in vivo, Cancer Res. 58 (1998) 5206-5215.

Denhardt DT, Chambers AF. Overcoming obstacles to metastasis--defenses against host defenses: osteopontin (OPN) as a shield against attack by cytotoxic host cells. J Cell Biochem 1994;56:48-51.

Denhardt DT and Guo X. Osteopontin: a protein with diverse functions. FASEB J 1993;7:1475-1482.

El-Tanani MK, Barraclough R, Wilkinson MC, Rudland PS. Regulatory region of metastasis-inducing DNA is the binding site for T cell factor-4. Oncogene 2001;20:1793-1797.

Feng B, Rollo EE, Denhardt DT. Osteopontin (OPN) may facilitate metastasis by protecting cells from macrophage NO-mediated cytotoxicity: evidence from cell lines down-regulated for OPN expression by a targeted ribozyme. Clin Exp Metastasis 1995;13:453-462.

Gardner HAR, Berse B, and Senger DR. Specific reduction in osteopontin synthesis by antisense RNA inhibits the tumorigenicity of transformed rat1 fibroblasts. Oncogene 1994;9:2321-2326.

Gunthert U, Hofmann M, Rudy W, Reber S, Zoller M, Haussmann I, Matzku S, Wenzel A, Ponta H, Herrlich P. A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. Cell 1991;65:13-24.

Jacks, T., L. Remington, B.O. Williams, E.M. Schmitt, S. Halachmi, R.T. Bronson, and R.A. Weinberg. Tumor spectrum analysis in p53-mutant mice. Curr. Biol. *4*:1-7, 1994.

Katagiri YU, Sleeman J, Fujii H, Herrlich P, Hotta H, Tanaka K, Chikuma S, Yagita H, Okumura K, Murakami M, Saiki I, Chambers AF, Uede T. CD44 variants but not CD44s cooperate with beta1-containing integrins to permit cells to bind to osteopontin independently of arginine-glycine-aspartic acid, thereby stimulating cell motility and chemotaxis. Cancer Res 1999;59:219-226.

S. Kasugai, Q. Zhang, C.M. Overall, J.L. Wrana, W.T. Butler, J. Sodek, Differential regulation of the 55 and 44 kDa forms of secreted phosphoprotein 1 (SPP-1, osteopontin) in normal and transformed rat bone cells by osteotropic hormones, growth factors and a tumor promoter, Bone Miner. 13 (1991) 235-250.

M.C. Kiefer, D.M. Bauer, P.J. Barr, The cDNA and derived amino acid sequence for human osteopontin, Nucleic Acids Res. 17 (1989) 3306.

S. Kon, M. Maeda, T. Segawa, Y. Hagiwara, Y. Horikoshi, S. Chikuma, K. Tanaka, M.M. Rashid, M. Inobe, A.F. Chambers, T. Uede, Antibodies to different peptides in osteopontin reveal complexities in the various secreted forms, J. Cell Biochem. 77 (2000) 487-498.

Liu XL, Wazer DE, Watanabe K, Band V. Identification of a novel serine protease-like gene, the expression of which is down-regulated during breast cancer progression. Cancer Res 1996;56:3371-9.

Matsumura Y, Tarin D. Significance of CD44 gene products for cancer diagnosis and disease evaluation. Lancet 1992;340:1053-1058.

Ratsch SB, Gao Q, Srinivasan S, Wazer DE, Band V. Multiple genetic changes are required for efficient immortalization of different subtypes of normal human mammary epithelial cells. Radiat Res 2001;155(1 Pt 2):143-150.

Rittling SR, Chen Y, Feng F, Wu Y. Tumor-derived osteopontin is soluble, not matrix associated. J Biol Chem 2001 (epub ahead of print).

Saitoh Y, Kuratsu J, Takeshima H, Yamamoto S, Ushio Y. Expression of osteopontin in human glioma. Its correlation with the malignancy. Lab Invest 1995;72:55-63. Schmits, R., J. Filmus, N. Gerwin, G. Senaldi, F. Kiefer, T. Kundig, A. Wakeham, A. Shahinian, C. Catzavelos, J. Rak, C. Furlonger, A. Zakarian, J.J. Simard, P.S. Ohashi, C.J. Paige, J.C. Gutierrez-Ramos, and T.W. Mak. CD44 regulates hematopoietic progenitor distribution, granuloma formation, and tumorigenicity. Blood *90*:2217-2233, 1997.

Schmits R, Filmus J, Gerwin N, Senaldi G, Kiefer F, Kundig T, Wakeham A, Shahinian A, Catzavelos C, Rak J, Furlonger C, Zakarian A, Simard JJ, Ohashi PS, Paige CJ, Gutierrez-Ramos JC, Mak TW. CD44 regulates hematopoietic progenitor distribution, granuloma formation, and tumorigenicity. Blood 1997 Sep 15;90(6):2217-2233.

D.R. Senger, B.B. Asch, B.D. Smith, C.A. Perruzzi, H.F. Dvorak, A secreted phosphoprotein marker for neoplastic transformation of both epithelial and fibroblastic cells, Nature 1983;302:714-715.

Senger DR, Peruzzi CA, and Papadopoulos A. Elevated expression of secreted phosphoprotein I (osteopontin, 2ar) as a consequence of neoplastic transformation. Anticancer Res 1989;48:1291-1299.

Senger DR, Wirth DF, and Hynes RO. Transformation-specific secreted phosphoproteins. Nature 1980;286:619-621.

Shanmugam V, Chackalaparampil I, Kundu GC, Mukherjee AB, Mukherjee BB, Altered sialylation of osteopontin prevents its receptor-mediated binding on the surface of oncogenically transformed tsB77 cells, Biochemistry 36 (1997) 5729-5738.

Su, L.K., K.W. Kinzler, B. Vogelstein, A.C. Preisinger, A.R. Moser, C. Luongo, K.A. Gould, and W.F. Dove. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. Science *256:*668-670, 1992.

Tanabe KK, Stamenkovic I, Cutler M, Takahashi K. Restoration of CD44H expression in colon carcinomas reduces tumorigenicity. Ann Surg 1995;222:493-501.

Weber GF, Ashkar S. Stress response genes - the genes that make cancer metastasize, J Mol Med 2000;78:404-408.

G.F. Weber, S. Ashkar, H. Cantor, Interaction between CD44 and osteopontin as a potential basis for metastasis formation, Proc. Assoc. Am. Physicians 109 (1997) 1-9.

Weber GF, Ashkar S, Glimcher MJ, Cantor H. Receptor-ligand interaction between CD44 and osteopontin (Eta-1). Science 1996;271:509-512.

Weber GF, Zawaideh S, Kumar VA, Glimcher MJ, Cantor H, Ashkar S. 2002. Phosphorylation-dependent interaction of osteopontin with its receptors regulates macrophage migration and activation. Journal of Leukocyte Biology, in press.

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Eta-1 (Osteopontin): An Early Component of Type-1 (Cell-Mediated) Immunity

Samy Ashkar,[1]* Georg F. Weber,[2,3]*†
Vassiliki Panoutsakopoulou,[2,4] Marie E. Sanchirico,[2,4]
Marianne Jansson,[2,4] Samer Zawaideh,[1] Susan R. Rittling,[5]
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Cell-mediated (type-1) immunity is necessary for immune protection against most intracellular pathogens and, when excessive, can mediate organ-specific autoimmune destruction. Mice deficient in Eta-1 (also called osteopontin) gene expression have severely impaired type-1 immunity to viral infection [herpes simplex virus–type 1 (KOS strain)] and bacterial infection (*Listeria monocytogenes*) and do not develop sarcoid-type granulomas. Interleukin-12 (IL-12) and interferon-γ production is diminished, and IL-10 production is increased. A phosphorylation-dependent interaction between the amino-terminal portion of Eta-1 and its integrin receptor stimulated IL-12 expression, whereas a phosphorylation-independent interaction with CD44 inhibited IL-10 expression. These findings identify Eta-1 as a key cytokine that sets the stage for efficient type-1 immune responses through differential regulation of macrophage IL-12 and IL-10 cytokine expression.

The development of cell-mediated (type-1) immune responses is necessary for protection against the growth of many infectious pathogens and, when excessive, can mediate autoimmune host tissue destruction. Although macrophage activation by microbial pathogens (*1, 2*) and foreign body reactions (*3*) are associated with type-1 immunity, the cellular and molecular events that imprint this response are not fully understood. An essential early step in this process is macrophage production of IL-12 at sites of infection, whereas early IL-10 production inhibits this response (*4*). Although IL-12 responses can be triggered by an interaction between the CD40 ligand on activated T cells and CD40 on macrophages (*4*), this interaction also induces the inhibitory IL-10 cytokine (*5, 6*), and its transient nature may not suffice for sustained IL-12 induction in vitro (*7*) or in vivo (*8*).

A gene product that may play an important role in the development of type-1 immunity is the T cell cytokine Eta-1 (for early T lymphocyte activation–1), also known as osteopontin (Opn) (*9*). The Eta-1 gene is expressed in T cells early in the course of bacterial infections (within 48 hours), and interaction of its protein product with macrophages can induce inflammatory responses (*10*). Genetic resistance to infection by certain strains of *Rickettsia* may depend on Eta-1–dependent attraction of monocytes into infectious sites and acquisition of bacteriocidal activity (*11*); the granulomatous responses characteristic of sarcoidosis and tuberculosis are associated with high levels of Eta-1 expression (*12*).

Granuloma formation in these human diseases is a cellular consequence of type-1 immunity (*12*), and sarcoid-type granulomas can be induced in mice after injection of polyvinyl pyrrolidone (PVP) (*13*). Because certain murine models of parasite-induced granulomas may reflect a mixture of type-2 and type-1 immunity (*6*), we first established the importance of IL-12–dependent type-1 immunity in this murine model of granuloma formation. An intense granulomatous response was provoked shortly after (subcutaneous) injection of PVP into C57BL/6 (+/+) but not C57BL/6 *nu/nu* strains of mice. This response was diminished by 70 to 80% in C57BL/6 IL-12$^{-/-}$ mice and was enhanced two- to threefold in C57BL/6 IL-10$^{-/-}$ mice (Fig. 1, A and B). Because C57BL/6 *nu/nu* mice coinjected with PVP and purified Eta-1 displayed a granulomatous reaction, this gene product can partially substitute for activated T lymphocytes in this setting (Fig. 1, A and B).

We then asked whether mice deficient in Eta-1 secondary to targeted gene mutation (*14*) formed granulomas after PVP injection. Eta-1$^{-/-}$ mice did not develop a detectable granulomatous response after challenge with PVP; the response was partially restored by coinjection of purified Eta-1 with PVP (Fig. 1, A and B). Histologic analysis of granulomas formed in Eta-1$^{+/+}$ mice and in Eta-1$^{-/-}$ mice reconstituted with purified Eta-1 revealed a similar macrophage-dominant cellular infiltrate: About 85% of granulomatous cells in both cases were Mac-1^{+}, whereas 5 to 10% were CD3^{+} T cells or B220^{+} B cells. BP-55^{+} neutrophils, which were only a minor component (1 to 2%) of granulomas in these mice, increased 5- to 10-fold in the granulomas of IL-10$^{-/-}$ mice (Fig. 1C). Eta-1$^{-/-}$ mice also displayed defective granulomatous responses to injection of collagen and latex, consistent with reports that human T cells resident in sterile granulomas have high expression of Eta-1 (*12*). Restimulation of lymph nodes draining subcutaneous sites of PVP injection in Eta-1$^{-/-}$ mice and control mice with PVP revealed impaired IL-12 and interferon-γ (IFN-γ) responses: The IL-12 response was reduced by ~95%, and the IFN-γ response of Eta-1$^{-/-}$ mice was reduced by 90% in comparison to Eta-1$^{+/+}$ controls (Fig. 1D).

We next defined the role of Eta-1 in the immune response to herpes simplex virus–type 1 (HSV-1) (KOS strain) infection. Eta-1$^{-/-}$ mice infected by HSV-1 [4 × 10^{6} plaque-forming units (PFU) via the cornea] did not develop a significant tuberculin-type delayed-type hypersensitivity (DTH) response after footpad challenge with HSV-1 (10^{5} PFU), in contrast to the strong DTH response of Eta-1$^{+/+}$ controls (Fig. 2A, left). Although the numbers of T cells and proportions of T cell subsets in the thymus and peripheral lymphoid tissues of Eta-1$^{-/-}$ mice were similar to Eta-1$^{+/+}$ littermates (*15*), defective antiviral DTH responses might reflect a subtle alteration in lymphocyte or macrophage development. We therefore tested the effects of acute in vivo depletion of Eta-1 with a neutralizing antibody. Administration of antibody to Eta-1 (LF-123) (*16*) immediately before and repeatedly after HSV-1 infection efficiently inhibited the DTH response upon rechallenge (Fig. 2A, right).

Corneal HSV-1 infection can also lead to a destructive type-1 autoimmune inflammatory reaction, herpes simplex keratitis (HSK), initiated by CD4 cells that recognize a viral peptide mimic (*17*). This inflammatory response depends on the production of IL-12 and is inhibited by IL-10 (*18*). Within 10 to 14 days after corneal HSV-1 infection, ~65% of control Eta-1$^{+/+}$ mice developed HSK, whereas HSV-1–infected Eta-1$^{-/-}$ mice did not readily develop this disease (Fig. 2B). Analysis of cells from the draining lymph nodes of virus-infected Eta-1$^{-/-}$ and Eta-1$^{+/+}$ mice indicated that they responded similarly to HSV-1 according to [^{3}H]thymidine incorporation after viral restimu-

[1]Laboratory for Skeletal Disorders and Rehabilitation, Department of Orthopedic Surgery, Children's Hospital, Harvard Medical School, 300 Longwood Avenue, Boston, MA 02115, USA. [2]Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute; [3]Department of Medicine; [4]Department of Pathology; Harvard Medical School, 44 Binney Street, Boston, MA 02115, USA. [5]Faculty of Arts and Sciences, Division of Life Sciences, Rutgers University, 604 Allison Road, NJ 08854, USA.

*These authors contributed equally to this work.
†Present address: Division of Radiation and Cancer Biology, New England Medical Center, 750 Washington Street, Boston, MA 02111, USA.
‡To whom correspondence should be addressed. E-mail: Harvey_Cantor@DFCI.harvard.edu

lation in vitro (*19*). However, draining lymph node cells from virally infected Eta-$1^{-/-}$ mice produced exaggerated amounts of IL-10 and IL-4 and reduced IL-12 in comparison with Eta-$1^{+/+}$ controls (Fig. 2C). In contrast with this sterile granulomatous response, IFN-γ levels were not reduced in Eta-$1^{-/-}$ mice after HSV-1 viral infection (*20*), consistent with an IL-12–independent pathway to IFN-γ production that may depend on virally induced IFN-α/β production (*21*).

We then investigated the ability of Eta-$1^{-/-}$ mice to mount a protective immune response after bacterial infection. The murine response to *Listeria monocytogenes* is an experimental cornerstone of our understanding of the early events leading to type-1 immunity after microbial infection (*1*) and depends on early macrophage production of IL-12 and downstream expression of IFN-γ (*22*). Eta-$1^{-/-}$ mice were defective in their ability to clear *L. monocytogenes* after systemic infection, similar to the defect in IL-$12^{-/-}$ mice (*23*) (see Web figure 1, available at www.sciencemag.org/feature/data/1046451.shl). Restimulation of spleen cells from Eta-$1^{-/-}$ and Eta-$1^{+/+}$ mice with heat-killed bacteria revealed that cells from the former mice had reduced IFN-γ responses: 25.5 ± 6.5 ng/ml of IFN-γ were produced by spleen cells from Eta-$1^{+/+}$ mice in comparison with 3.2 ± 1.2 ng/ml of IFN-γ from Eta-$1^{-/-}$ mice (*24*).

Thus, Eta-1 expression may affect type-1 immunity through regulation of the IL-12 and IL-10 cytokine ratio. To define the effect of Eta-1 on IL-10 and IL-12 production by macrophages in vitro, we incubated resident peritoneal macrophages with increasing concentrations of purified Eta-1 in serum-free medium (Fig. 3A). This resulted in the secretion of as much as 400 pg/ml of IL-12 at 48 hours, whereas IL-10 production was not detected (Fig. 3, A and B). The failure of Eta-1 to induce IL-10 was somewhat surprising because other cytokines that activate macrophages, including tumor necrosis factor–α, IL-1, IL-2, IL-3, and IL-6, all stimulate IL-10 secretion (*25*), and lipopolysaccharide (LPS) stimulation of these resident peritoneal macrophages induced both IL-12 (~250 pg/ml) and IL-10 (~100 pg/ml) (Fig. 3B). Further analysis showed that Eta-1 actively suppressed the LPS-dependent IL-10 response of resident peritoneal macrophages by 80 to 95% (Fig. 3C).

The interaction of Eta-1 with macrophages is mediated through two functional receptors. Engagement of CD44 mediates chemotactic migration (*26*), and interaction with $\alpha_v\beta_3$ integrin causes haptotaxis, adhesion, and spreading (*10, 27*). We asked which receptor was responsible for the regulation of macrophage cytokine production by Eta-1. Induction of IL-12 is inhibited by GRGDS (*28*) peptide (but not GRADS peptide) (*29*) and by antibody to the integrin β_3 subunit (but not by antibody to CD44), and macrophages from CD44$^{-/-}$ mice

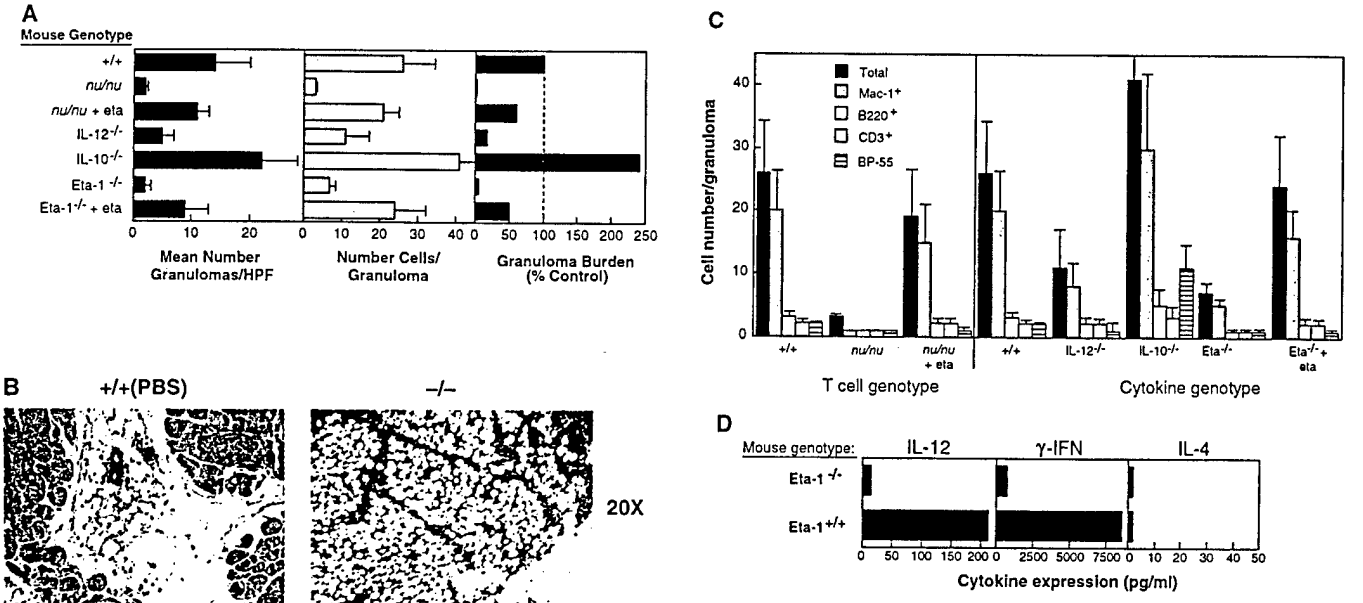

Fig. 1. Granuloma formation in cytokine-deficient mice. Granulomas were measured 5 days after subcutaneous injection of PVP into mice carrying the indicated mutations of cytokine genes (*38*). (**A**) Results are expressed as the mean number of granulomas per high-power field (HPF) (×200 magnification) (left); as the mean number of cells per granuloma after examination of 50 to 80 HPF per mouse (middle); and as the product of these two indices, shown as granuloma burden (right). Error bars indicate 1 SEM. (**B**) Photomicrographs at indicated magnifications showing histologic analysis of tissue sections at PVP injection sites of Eta-$1^{+/+}$ (left) or Eta-$1^{-/-}$ (right) mice showing granulomatous infiltrations of mononuclear cells in subcutaneous dermal and subdermal areas 5 days after injection of PVP, PBS, or PVP + 5 μg purified Eta-1 (*40*). (**C**) Analysis of surface antigens expressed by cells within granulomas in the indicated mouse strains was done with monoclonal antibodies to Mac-1, B220, CD3, and BP-55 (a neutrophil marker). Error bars indicate 1 SEM. (**D**) Cytokine expression by cells from lymph nodes draining the site of granulomas 5 days after PVP injection was measured 48 hours after incubation with PVP (2×10^6 cells per well). Data are representative of three independent experiments.

(*30*) display an unimpaired IL-12 response (Fig. 4, A to C). Moreover, Eta-1–dependent induction of IL-12 secretion from macrophages was not due to contamination with endotoxin: *Limulus* lysate analysis indicated that purified Eta-1 contained <1 ng/g of endotoxin, and the IL-12 response of macrophages from C3H.HeJ mice (which are defective in endotoxin receptor-mediated signaling) was not obviously impaired in comparison to other strains (Fig. 4C). In contrast to IL-12 induction, inhibition of IL-10 depends on engagement of the CD44 receptor: Eta-1–dependent inhibition of IL-10 is blocked by antibody to CD44 but not by antibody to integrin β_3, and macrophages from CD44$^{-/-}$ mice are resistant to Eta-1 inhibition of the IL-10 response (Fig. 4, A to C). To further characterize the RGD-dependent interaction with the macrophage integrin receptor, we analyzed fragments from an Eta-1 Lys-C digest and identified a proteolytic fragment from the NH_2-terminal portion of Eta-1, which contains the integrin binding site (termed NK10) that is sufficient to induce macrophage IL-12 expression (Fig. 4A).

Eta-1 is secreted in nonphosphorylated and phosphorylated forms (*31*). Phosphorylation may allow Eta-1 to associate with the cell surface rather than the extracellular matrix (*32*), through a contribution to integrin binding. In contrast, serine phosphorylation of recombinant Eta-1 is not required for CD44-dependent interactions leading to chemotactic migration (*26*). We investigated whether phosphorylation of Eta-1 might affect its ability to regulate IL-12 and IL-10 expression. Dephosphorylation of purified, naturally produced Eta-1 abolished IL-12 stimulatory activity; phosphorylation of recombinant Eta-1 at specific sites restored activity (*33*) (see Web figure 2, available at www.sciencemag.org/feature/data/1046451.shl). Although recombinant Eta-1–lacking phosphate groups could not induce IL-12, this molecule retained inhibitory activity for the macrophage IL-10 response (*33*). Thus, serine phosphorylation can provide molecular information that regulates the biological activity of a secreted protein.

Our data indicate that expression of Eta-1 represents an essential early step in the pathway that leads to type-1 immunity. Previous studies have established the importance of macrophage production of IL-12 in this pathway (*1, 4, 22*). Our experiments suggest that production of Eta-1 by activated T cells is an essential proximal event that potentiates the macrophage IL-12 response through integrin engagement and dampens the IL-10 response through CD44 engagement, leading to up-regulation of type-1 cytokines. The latter inhibitory effect on IL-10 may account for enhanced granulomatous responses of CD44$^{-/-}$ mice (*30*) and the finding that impairment of the granuloma response noted here is somewhat greater than might be anticipated from the response of IL-12–deficient mice (Fig. 1). These findings fill a logical gap in our understanding of the early molecular events that lead to type-1 immunity. Although down-regulation of CD40 ligand expression by IFN-γ and soluble CD40 occurs within 24 hours after viral infection, IL-12 is detected in serum over the next 7 to 10 days (*8*). Our experiments suggest that replacement of the CD40L signal by Eta-1 may potentiate the IL-12 response while dampening the IL-10 activity to allow full maturation of type-1 immunity, as judged by cellular responses and expression of downstream effector cytokines such as IFN-γ. The ability of an antigen to induce Eta-1 production after T cell receptor ligation may thus determine the ensuing duration and intensity of type-1 immune responses. Eta-1 imprinting of the IL-12 and IL-10 response after appropriate peptide stimulation (*34*) may also increase the likelihood of autoimmune sequelae as shown here (Fig. 2), through pathways that do not invariably require IFN-γ (*35*).

Eta-1–dependent regulation of two early cytokine checkpoints that dictate development of type-1 or type-2 immunity also suggests new therapeutic approaches to several diseases. Eta-1 analogs that mediate CD44-dependent inhibition of IL-10 may inhibit sepsis in burn patients (*36*), and Eta-1 antagonists may ameliorate the clinical course of bacterial arthritis (*37*). Engineered forms of

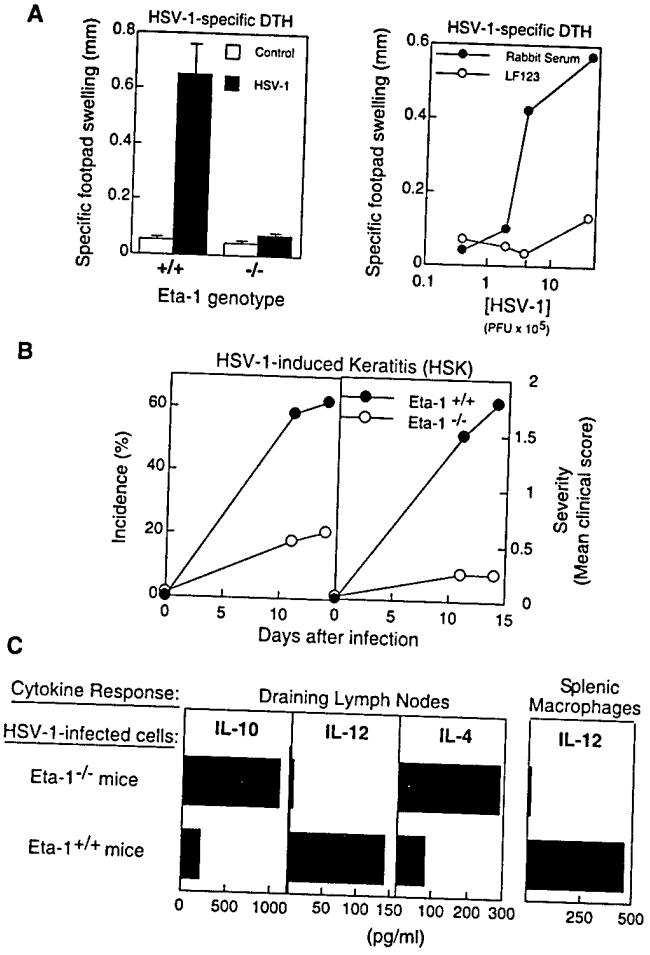

Fig. 2. The role of Eta-1 in immunity to HSV-1 (KOS strain). (**A**) (Left) Defective HSV-1–specific DTH responses in Eta-1$^{-/-}$ mice. C57BL/6 × 129 strain mice with or without a targeted disruption of the Eta-1 gene (–/–) or controls (+/+) were infected in the right eye with 4 × 10^6 PFU of HSV-1 (KOS) and challenged 5 days later in the left footpad with 1 × 10^5 PFU of UV-inactivated HSV-1 (KOS). The right (control) and left (HSV-1) footpads of each mouse were measured 24 hours later with a micrometer. Each data point represents the mean and standard error (error bars) of three mice per group. (Right) Inhibition of the anti–HSV-1 DTH response by acute depletion of Eta-1. The neutralizing antisera LF-123 (*16*) or control normal rabbit serum were injected at 25 μg per dose per day, starting 2 days before injection. On day 0, mice were infected with HSV-1 (KOS) and rechallenged 5 days later. The right and left footpads of each mouse were measured 24 hours after rechallenge, and specific swelling (left versus right footpad) is shown. (**B**) Development of HSK in Eta-1$^{-/-}$ mice. The right eyes of Eta-1$^{-/-}$ and Eta-1$^{+/+}$ mice were infected with 4 × 10^6 PFU of HSV-1 (KOS), and disease was assessed on days 11 and 14 after infection, as described (*17*). The severity of clinical stromal keratitis was scored on the basis of the percentage of corneal opacity: ≤25%, 1; ≤50%, 2; ≤75%, 3; and 75 to 100%, 4. Each point represents at least 16 mice and is the mean of three independent experiments. (**C**) Differential cytokine profile of draining lymph node cells and splenic macrophages from Eta-1$^{+/+}$ or Eta-1$^{-/-}$ mice after infection with HSV-1. Cytokine levels after restimulation of draining lymph node cells (from mice 15 days after HSV-1 infection in vivo) by 4 × 10^7 PFU of UV-inactivated HSV-1 using 48-hour supernatants were determined by ELISA (*19*). Viral restimulation of mixtures of purified lymph node T cells from virus-infected donors and syngeneic (nonimmune) adherent cells yielded less than one-third of the IL-10 response of mixtures of immune T cells and macrophages from draining lymph nodes of infected donors (*20*). The proliferative response of lymph node cells from HSV-1–infected Eta-1$^{+/+}$ and Eta-1$^{-/-}$ mice measured by [^{3}H]thymidine incorporation at 72 hours was 20.9 × 10^3 and 18.7 × 10^3 cpm, respectively.

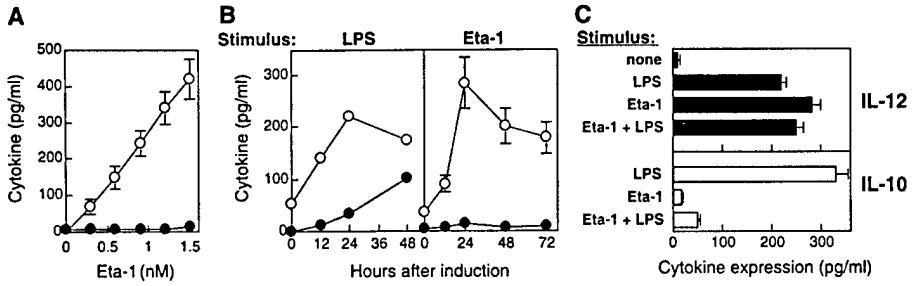

Fig. 3. Differential regulation of macrophage IL-12 and IL-10 responses by purified Eta-1. **(A)** Dose-dependent induction of IL-12 (open circles), but not IL-10 (solid circles) production, from macrophages by Eta-1. Resident peritoneal macrophages obtained from C57BL/6 mice (*41*) were incubated for 48 hours (5×10^5 macrophages per milliliter) with purified Eta-1 (*40*), and IL-10 and IL-12 p70 concentrations in the supernatant were determined by ELISA. Assays were done in quadruplets, and each point represents the mean and standard error (error bars) of three independent experiments. **(B)** Time course of IL-12 (open circles) p70 and IL-10 (solid circles) expression by resident peritoneal macrophages (5×10^5 macrophages per milliliter) after incubation with 5 nM Eta-1 or LPS (30 ng/ml). Assays were performed in quadruplets, and each data point represents the mean and standard error (error bars) of two independent experiments. **(C)** Inhibitory effect of Eta-1 on macrophage IL-10 production. Macrophages were activated with LPS (30 ng/ml) for 1 hour before addition of Eta-1 (5 nM) for an additional 48 hours and consecutive measurement of IL-12 and IL-10 by ELISA. Assays were performed in quadruplets, and each point represents the mean and standard error (error bars) of two independent experiments.

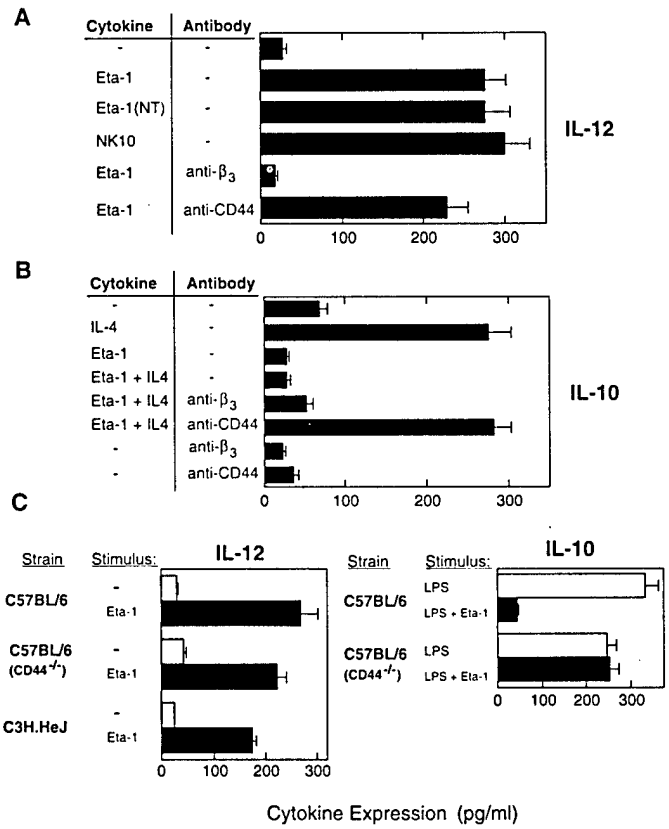

Fig. 4. Regulation of macrophage IL-12 and IL-10 expression by distinct Eta-1 receptors. **(A)** Secretion of IL-12 by macrophages is mediated by a 10-kD peptide (NK10) derived from the NH_2-terminal fragment of Eta-1 (*42*) and is inhibited by a blocking antibody to integrin β_3 (1 μg/ml), but is unaffected by antibody to CD44 (1 μg/ml). Error bars indicate 1 SEM. **(B)** Induction of IL-10 production by IL-4 (500 U/ml) in the presence or absence of purified Eta-1 (5 nM) and the effects of antibodies to CD44 [Km81 purified from TIB241 (*26*)] and the β_3 integrin (2C9.G2, PharMingen) are shown. Error bars indicate 1 SEM. **(C)** Production of IL-12 and IL-10 in response to Eta-1 by peritoneal macrophages from C57BL/6 mice, C57BL/6 mice that are deficient in CD44 gene expression (C57BL/6–CD44$^{-/-}$), and cells from C3H.HeJ mice. Mean values and standard errors (error bars) from at least four data points are shown.

Eta-1 that imprint type-1 responses after immunization may also be valuable components of viral and cancer vaccines.

References and Notes

1. E. R. Unanue, *Immunol. Rev.* **158**, 11 (1997).
2. R. Medzhitov and C. Janeway, *Semin. Immunol.* **10**, 351 (1998).
3. P. Matzinger, *Semin. Immunol.* **10**, 399 (1998).
4. C. M. Scheicher, M. Mehlig, H. P. Dienes, K. Reske, *Eur. J. Immunol.* **25**, 1566 (1995); S. E. Macatonia *et al.*, *J. Immunol.* **154**, 5071 (1995); K. M. Murphy, *Curr. Opin. Immunol.* **10**, 226 (1998).
5. F. Koch *et al.*, *J. Exp. Med.* **184**, 741 (1996).
6. A. O'Garra, *Immunity* **8**, 275 (1998).
7. F. Ria, G. Penna, L. Adorini, *Eur. J. Immunol.* **28**, 2003 (1998).
8. M. D. Sharma, M. L. de Moraes, F. Zavala, C. Pontoux, M. Papiernik, *J. Immunol.* **161**, 5357 (1998).
9. R. Patarca *et al.*, *J. Exp. Med.* **170**, 145 (1989); G. F. Weber, S. Ashkar, H. Cantor, *Proc. Assoc. Am. Physicians* **109**, 1 (1997); S. R. Rittling and D. T. Denhardt, *Exp. Nephrol.* **7**, 103 (1999).
10. R. P. Singh, R. Patarca, J. Schwartz, P. Singh, H. Cantor, *J. Exp. Med.* **171**, 1931 (1990); X. Q. Yu *et al.*, *Proc. Assoc. Am. Physicians* **110**, 50 (1998); D. T. Denhardt and M. Noda, *J. Cell. Biochem. Suppl.* **30/31**, 92 (1998).
11. R. Patarca, R. A. Saavedra, H. Cantor, *Crit. Rev. Immunol.* **13**, 225 (1993); T. J. Jerrells and J. V. Osterman, *Infect. Immun.* **31**, 1014 (1981).
12. G. J. Nau *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 6414 (1997); A. W. O'Regan *et al.*, *J. Immunol.* **162**, 1024 (1999).
13. C. van den Bogert *et al.*, *Virchows Arch.* **51**, 39 (1986).
14. C57BL/6 × 129/SV Eta-1$^{-/-}$ mice generated as described by S. R. Rittling *et al.* [*J. Bone Miner. Res.* **13**, 1101 (1998)] were compared to either Eta-1$^{+/+}$ littermates or age-matched C57BL/6 × 129/SV mice as controls.
15. T and B cell subsets in Eta-1$^{-/-}$ and Eta-1$^{+/+}$ littermates were as follows: C57BL/6 × 129 Eta-1$^{+/+}$ spleen, 93.7×10^6 total cells (30.8% CD3, 19.8% CD4, 11% CD8, and 49.7% B220); C57BL/6 × 129 Eta-1$^{-/-}$ spleen, 82.6×10^6 cells (27.8% CD3, 18.8% CD4, 9.0% CD8, and 55.5% B220); C57BL/6 × 129 Eta-1$^{+/+}$ lymph node, 32.0×10^6 cells (82.4% CD3, 42.8% CD4, 34.2% CD8, and 12.8% B220); and C57BL/6 × 129 Eta-1$^{-/-}$ lymph node, 21.9×10^6 cells (82.8% CD3, 49.3% CD4, 28.4% CD8, and 11.2% B220). T cells from Eta-1$^{-/-}$ and Eta-1$^{+/+}$ mice expressed levels of CD44 and CD62 that were not distinguishable. T cell expansion followed by apoptosis after superantigen (50 μg of staphylococcal enterotoxin B) intraperitoneal injection into Eta-1$^{-/-}$ and Eta-1$^{+/+}$ mice was indistinguishable at 3 days: +/+ $V_\beta8^+$ CD4 cells (percentage of total spleen) increased from 3.6 to 5%; −/− $V_\beta8^+$ CD4 cells increased from 3.2 to 5.5%; +/+ $V_\beta6^+$ CD4 cells increased from 2.3 to 2.6%; −/− $V_\beta6^+$ CD4 cells increased from 2.5 to 2.6%. Expression of IL-2 by lymph node and spleen T lymphocytes from Eta-1$^{-/-}$ and Eta-1$^{+/+}$ littermates in response to immobilized antibody to CD3 was also indistinguishable between the C57BL/6 × 129/SV Eta-1$^{-/-}$ and C57BL/6 × 129/SV Eta-1$^{+/+}$ mice.
16. L. W. Fisher, J. T. Stubbs, M. F. Young, *Acta Orthop. Scand.* **66**, 61 (1995).
17. Z.-S. Zhao, F. Granucci, L. Yeh, P. A. Schaffer, H. Cantor, *Science* **279**, 1344 (1998); A. C. Avery *et al.*, *Nature* **376**, 431 (1995).
18. J. W. Streilein, M. R. Dana, B. R. Ksander, *Immunol. Today* **18**, 443 (1997); M. Daheshia, N. Kuklin, S. Kanangat, E. Manickan, B. T. Rouse, *J. Immunol.* **159**, 1945 (1997).
19. HSV-1 stimulation of lymph node cells and splenic macrophages: The right superficial cervical draining lymph nodes of Eta-1$^{-/-}$ mice and Eta-1$^{+/+}$ littermate controls were harvested 15 days after infection of the right eye with 4×10^6 PFU of HSV-1 (KOS). Cells from these lymph nodes (2×10^6 cells per well) were incubated in the presence of 4×10^7 PFU of ultraviolet (UV)–inactivated HSV-1 (KOS). Supernatants harvested 48 hours later were tested for IL-10 and IL-12 p40 cytokine by sandwich enzyme-linked immunosorbent assay (ELISA) using OptEIA antibody sets (PharMingen, La Jolla, CA). IL-4 was measured after stimulation of draining lymph node cells by plate-bound antibody to CD3.
20. S. Ashkar *et al.*, data not shown.
21. A. Oxenius, U. Karrer, R. M. Zinkernagel, H. Hengartner, *J. Immunol.* **162**, 965 (1999); L. P. Cousens *et al.*, *J. Exp. Med.* **189**, 1315 (1999).
22. C. S. Tripp, S. F. Wolf, E. R. Unanue, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 3725 (1993); C. S. Tripp, M. K. Gately, J. Hakimi, P. Ling, E. R. Unanue, *J. Immunol.* **152**, 1883 (1994); C. S. Tripp, O. Kanagawa, E. R. Unanue, *J. Immunol.* **155**, 3427 (1995).
23. *Listeria* infection and cytokine production were as followed. Virulent *L. monocytogenes* [strain 1778, American Type Culture Collection (ATCC) designation 43251] was grown in trypticase soy broth (Becton-Dickinson), and 10^3 colony-forming units (CFU),

a sublethal dose for this strain of *L. monocytogenes*, were injected intravenously. The titer of viable bacteria in the inoculum and in organ homogenates was determined by plating 10-fold serial dilutions on trypticase soy agar plates. Eta-$1^{-/-}$ mice contained liver-associated *Listeria*-infected cysts that were apparent 4 to 5 days after infection. Plates were incubated at 37°C, and the numbers of CFU were counted after 24 hours.

24. Spleen cells (4×10^6/ml) from four to five C57BL/6 × 129 Eta-$1^{+/+}$ or four to five C57BL/6 × 129 Eta-$1^{-/-}$ mice that had been intravenously inoculated 5 days earlier with 10^3 CFU were stimulated with heat-killed *L. monocytogenes* (2×10^8 CFU/ml) 96 hours before IFN-γ measurement by an OptEIA ELISA kit (PharMingen).
25. P. Stordeur *et al.*, *Mol. Immunol.* **32**, 233 (1995); P. Stordeur and M. Goldman, *Int. Rev. Immunol.* **16**, 501 (1998).
26. G. F. Weber, S. Ashkar, M. J. Glimcher, H. Cantor, *Science* **271**, 509 (1996).
27. L. Liaw *et al.*, *J. Clin. Invest.* **95**, 713 (1995); D. R. Senger *et al.*, *Am. J. Pathol.* **149**, 293 (1996).
28. Addition of 5 mM GRGDS peptide but not GRADS peptide (*29*) to cultures of peritoneal macrophages (*38*) resulted in an 80% reduction of the IL-12 response after LPS stimulation.
29. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; E, Glu; G, Gly; L, Leu; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; and T, Thr.
30. R. Schmits *et al.*, *Blood* **90**, 2217 (1997).
31. T. Kubota *et al.*, *Biochem. Biophys. Res. Commun.* **162**, 1453 (1989); A. F. Chambers, E. I. Behrend, S. M. Wilson, D. T. Denhardt, *Anticancer Res.* **12**, 43 (1992); E. S. Sorensen and T. E. Petersen, *Biochem. Biophys. Res. Commun.* **198**, 200 (1994); H. Salih, S. Ashkar, L. C. Gerstenfeld, M. J. Glimcher, *J. Biol. Chem.* **272**, 13966 (1997).
32. M. Nemir, M. W. DeVouge, B. B. Mukherjee, *J. Biol. Chem.* **264**, 18202 (1989); B. Ek-Rylander, M. Flores, M. Wendel, D. Heinegard, G. Andersson, *J. Biol. Chem.* **269**, 14853 (1994).
33. Dephosphorylation of Eta-1 is as follows. Five milligrams of purified Eta-1 was dephosphorylated using 6 U (60 U/mg) of type-II potato acid phosphatase (Sigma) as described (*39*). Phosphorylation of recombinant Eta-1:glutathione *S*-transferase (GST)–Eta-1 (5 mg) was phosphorylated as described [S. Ashkar, J. L. Schaffer, E. Salih, L. C. Gerstenfeld, M. J. Glimcher, *Ann. N.Y. Acad. Sci.* **760**, 296 (1995); (*27*)]. Phospho–amino acid analysis of the recovered protein revealed a phosphoserine content of 16 mol of phosphate per 1 mol of protein and 0.8 mol of phosphothreonine per 1 mol of protein.
34. S. L. Constant and K. Bottomly, *Annu. Rev. Immunol.* **15**, 297 (1999).
35. B. M. Segal, B. K. Dwyer, E. M. Shevach, *J. Exp. Med.* **187**, 537 (1998).
36. A. Lyons, J. L. Kelly, M. L. Rodrick, J. A. Mannick, J. A. Lederer, *Ann. Surg.* **226**, 450 (1997); J. L. Kelly, A. Lyons, C. C. Soberg, J. A. Mannick, J. A. Lederer, *Surgery* **122**, 146 (1997).
37. Z. Yin *et al.*, *Arthritis Rheum.* **40**, 1788 (1997).
38. PVP-dependent granulomas were formed as follows. C57BL/6 (+/+), C57BL/6 *nu/nu*, C57BL/6–IL-$12^{-/-}$, C57BL/6 × 129 Eta-$1^{+/+}$, and C57BL/6 ×129 Eta-$1^{-/-}$ mice were injected subcutaneously above the right hind limb with 500 μl of 0.5% PVP; C57BL/6 *nu/nu* and Eta-$1^{-/-}$ mice were coinjected with this inoculum of PVP and 10 μg of Eta-1 and purified as described below. After 5 days, mice were killed, tissue was extracted for histologic analysis, and local lymph nodes were obtained for cytokine expression. Samples were fixed in 10% buffered formalin and embedded in paraffin; 4- to 5-μm serial sections were stained with hematoxylin and eosin, and images were captured with a Sony DXC-970MD video camera and Optima 5.2 Histomorphometric analysis software.
39. S. Ashkar *et al.*, *Biochem. Biophys. Res. Comm.* **191**, 126 (1993).
40. Recombinant GST–Eta-1 fusion protein derived from *Escherichia coli* was digested with factor Xa and purified by affinity chromatography [S. Ashkar, M. J. Glimcher, R. A. Saavedra, *Biochem. Biophys. Res. Commun.* **194**, 274 (1993); (*39*)]. Briefly, native Eta-1 was isolated from MC3T3E1 cells or Ar5v T cells after concentration in PBS using a Millipore tangential flow system, applied to a Millipore LC100 equipped with a DEAE-Memsep 1000 cartridge, and developed in a discontinuous gradient of 0 to 1 M NaCl in phosphate buffer (pH 7.4). Eta-1–containing fractions were pooled (the major Eta-1 peak eluted at 0.26 M salt), concentrated by ultrafiltration, chromatofocused on mono P columns (Pharmacia) at pH 8.2, developed with polybuffer 74 (Pharmacia), and eluted from monobeads at pH 4.6. The eluted protein was judged to be pure by several criteria, including SDS electrophoresis and amino acid sequence analysis (NH_2-terminal and internal peptide analysis). Mass spectroscopic analysis revealed a peak centered around a mass of 35,400 daltons that was highly phosphorylated (11 mol of phosphate per 1 mol of protein), O-glycosylated but not N-glycosylated, and without measurable sulfate.
41. Resident peritoneal macrophages obtained by peritoneal lavage with phosphate-buffered saline (PBS) were treated with red cell lysis buffer and incubated (10^5 macrophages per 100 μl) for 2 hours. The adherent fraction was incubated with 5 nM Eta-1, LPS (30 ng/ml), or recombinant IL-4 (500 U/ml), or as indicated. Supernatant IL-10 or IL-12 p70 was assayed with commercial ELISA kits (R&D Systems, Minneapolis, MN), tested for viability by propidium iodide (>98%), and stained with fluorescein-conjugated antibody to Mac-1 (>98%). Blocking antibody to integrin β_3 was from PharMingen [J. F. Schultz and D. R. Armant, *J. Biol. Chem.* **270**, 11522 (1995)], and antibody to CD44, KM81 (ATCC), was used to block the interaction between CD44 and Eta-1 (*16*).
42. Although partial tryptic, chemotryptic, or Asp-N endopeptidase digestion of Eta-1 did not reveal an active peptide, a 10-kD fragment isolated from a Lys-C digest [NH_2-terminal sequence QETLPSN (*29*)] was active and predicted to terminate at the thrombin cleavage site. This 10-kD fragment contained ~5 mol of phosphate per 1 mol of peptide at seven potential phosphorylation sites.
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HYPOTHESIS

Georg F. Weber · Samy Ashkar

Stress response genes: the genes that make cancer metastasize

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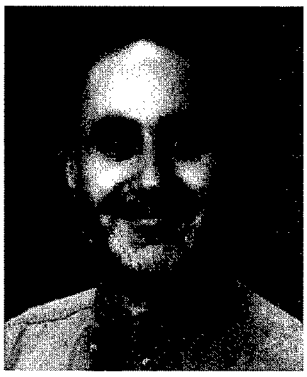
GEORG F. WEBER received his M.D. in 1988 from the University of Würzburg, Germany. Following postdoctoral studies in biochemistry, cancer pharmacology, and immunology he served on the faculty of the Dana-Faber Cancer Institute and Harvard Medical School from 1993 to 1999 before becoming Assistant Professor at Tufts University School of Medicine and the New England Medical Center. His research focuses on immunology and cancer metastases.



SAMY ASHKAR has served on the faculty of Harvard Medical School for the past 10 years. His laboratory current interests include the structure-function analysis of extracellular matrix molecules and the role of the extracellular matrix in tissue specific patterning. He and Dr. Weber have been collaborating for the past 7 years and have coauthored several papers.

G.F. Weber (✉)
Department of Cancer Immunology and AIDS,
Dana-Farber Cancer Institute, Department of Medicine,
Harvard Medical School, Boston, Massachusetts, USA
e-mail: weber@mbcrr.harvard.edu

S. Ashkar
Laboratory for Skeletal Disorders and Rehabilitation,
Children's Hospital, Department of Orthopedic Surgery,
Harvard Medical School, Boston, Massachusetts, USA

Present address:
G.F. Weber, Department of Radiation Oncology,
New England Medical Center and Tufts University Medical School,
750 Washington Street, NEMC #824, Boston, MA 02111, USA,
e-mail: Gweber@lifespan.org,
Tel.: +1-617-6369013, Fax: +1-617-6361766

Abstract Cancer is characterized by dysregulated growth control, overcoming of replicative senescence, and metastasis formation. The topology of cancer spread is mediated by a set of developmentally nonessential genes which are physiologically involved in stress responses, inflammation, wound healing, and neovascularization. The function of these gene products is extensively modified posttranscriptionally. In cancer, metastasis genes are dysregulated at the levels of expression or splicing. These genes constitute a unique group of cancer-related biomolecules.

Key words Cancer · Metastasis · Stress response · Posttranslational modification · Knockout mouse

Introduction

What are the traits that make a killer? This question has intrigued not only fans of detective stories but is also most prominent in the minds of cancer researchers. Here the characteristics of the killer are dysregulated growth control, overcoming of replicative senescence, and metastasis formation. The division of normal cells is tightly controlled by dependence on checkpoints, which are pauses during the cell cycle in which the fidelity of DNA replication and chromosome segregation are monitored. It is regulated by proto-oncogenes, incorporating genes for growth factors, their receptors, and associated intracellular signal transduction molecules. Antagonistic to oncogenes are tumor suppressor genes which normally provide the brakes on cell proliferation. In contrast, cancer is independent of these control mechanisms. Even with defective growth control, however, a cell could never form a tumor of substantial size because, unless it were a germline cell, it would be subject to replicative senescence, an aging process that proceeds with the number of cell divisions and in extreme cases may lead to a state of crisis. A unique role in overcoming replicative senescence is played by the enzyme telomerase, which is expressed in virtually all tumor cells but is ab-

sent from most normal cells. It prevents telomere shortening with increased number of cell divisions, which would eventually cause genomic instability. Mutator genes which encode DNA repair enzymes might be more accurately referred to as meta-oncogenes because their defects give rise to mutations in oncogenes and tumor suppressor genes. Finally, most cells, with the exception of blood and immune cells, grow anchored in their microenvironment whereas cancer cells of particular tissue origin metastasize to specific target organs. The ability of cancer to disseminate throughout the body also sets it apart from benign tumors. However, the classical cancer genes conspicuously do not account for metastasis formation, and current paradigms of cancer have not yet incorporated metastasis genes as a unique group of genes that contributes to the malignant phenotype.

The gene products of stress responses mediate metastasis formation: lessons from knockout mice

The topology of metastasis formation is mediated by the potpourri of homing receptors on the tumor cell surface (Fig. 1) and their ligands and is widely believed to have its physiological correlate in morphogenesis during embryonic development. This would imply that the deficiency of individual metastasis genes should cause defective formation of the relevant target organ. Unexpectedly, knockout mice in which individual genes known to participate in tumor spread were disrupted proved to be fertile and developmentally normal (Table 1). This raises the question: What is the physiological process that goes astray in cancer dissemination?

Despite their diversity, metastasis-associated gene products have several features in common. They comprise a set of genes which physiologically mediate stress responses, including inflammation, wound healing, and neovascularization. Consistently the defects observed in the relevant gene targeted mice are impairments in these areas. This insight resolves some of the paradoxes of metastasis research. In contrast to morphogenesis, invasiveness and tissue damage are in keeping with the normal functions of host defenses that are executed by macrophages and lymphocytes in stress situations. Homing to and expansion in the lymphoid system, typically the first target in metastatic spread, corroborate the notion that cancer metastasis is based on mechanisms normally employed by immunocytes [1]. Differentiation of immune cells proceeds in the context of their tissue of residence, and lymphocytes from Peyer's patches are therefore distinct from cutaneous lymphocytes, and Kupffer cells are distinct from alveolar macrophages. Recognition of topology is encoded in the surface molecules of immune cells, and organ preference by cancer may be derived from this principle.

Table 1 Genes that mediate cancer spread are developmentally nonessential. Cancer dissemination is induced by a group of homing receptors, their ligands, and proteinases in conjunction with their associated signal transduction molecules. These gene products do not play a critical role in organ development or fertility but are necessary for stress responses. Knockout mice have been generated for multiple metastasis associated genes and uniformly show these characteristics. Various integrins have also been linked to metastasis formation but most integrin gene knockouts display developmental defects. This may be due to the loss of multiple receptors after deletion of individual integrin genes. Furthermore, some intergin gene products serve dual roles in stress responses and development (*DTH* delayed type hypersensitivity, *MMP* matrix metalloproteinase, *uPAR* receptor for urokinase-type plasminogen activator)

Gene	Types of cancer	Knockout mouse
Receptors		
uPAR	Prostate cancer, breast cancer [24], gastric carcinoma [25], brain tumors [26]	Defect in leukocyte recruitment and adhesion [8]
CD44	Lymphomas [27], sarcomas [28], colon cancer [29], breast cancer [30]	Excessive granuloma formation [9]
L-selectin	Lymphoma [31]	No DTH to cutaneous antigens [10]
LFA-1	lymphoma [32]	Impaired immune response to alloantigens [11]
ICAM-1	Melanoma [33], lymphoma [34], liver carcinoma [35]	Granulocytosis, diminished DTH, impaired neutrophil homing [12, 13]
IAP (CD47)	Ovarian cancer [36]	Impaired granulocyte activation [14]
Ligands		
Osteopontin	Breast cancer [37], osteosarcoma [38]	Defective wound healing, absence of DTH [15, 16]
Thrombospondin-1	Breast cancer [39], pancreas cancer [40]	Susceptibility to pneumonia [17]
sE-selectin	Gastric cancer [41], breast cancer [42], head and neck cancer [43]	reduced stable adhesion of Leukocytes in inflamed microvasculature [18, 19]
P-selectin	Breast cancer [44], colon cancer [45]	Impaired recruitment of immune cells [20]
Proteinases		
Stromelysin-3 (MMP-3)	Breast cancer [46]	Impaired wound healing [21]
Matrilysin (MMP-7)	Colon cancer [47]	Decreased antimicrobial activity, defective reepithelialization in wounded trachea [22, 23]
Macrophage elastase (MMP-12)	Glioma [48]	Impaired macrophage recruitment [21]

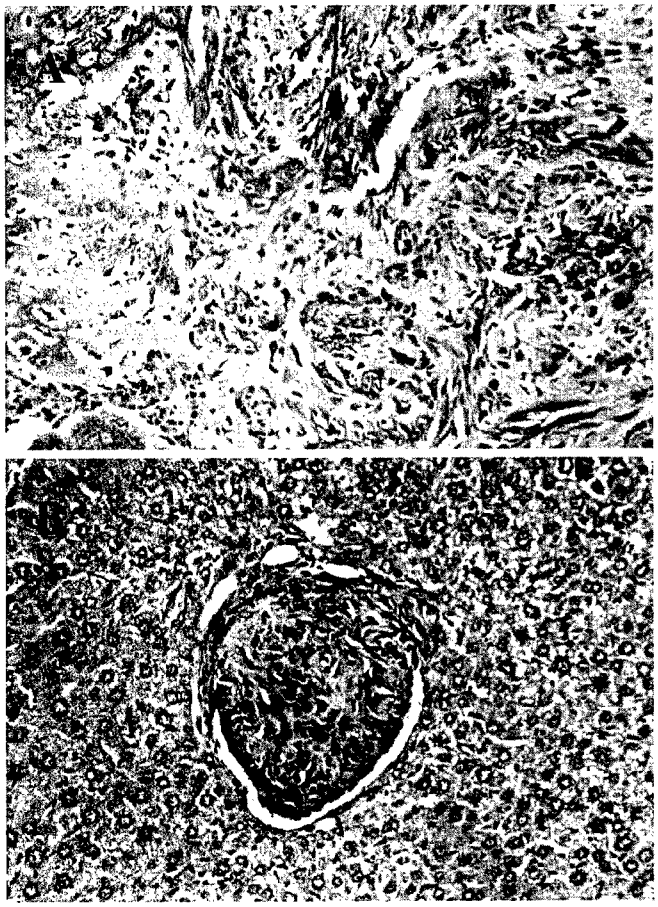


Fig. 1A,B CD44 is essential for metastasis formation by osteosarcoma. C57BL/6 mice with the tm1 point mutation of the *p53* gene are susceptible to osteosarcomas which disseminate to liver and lungs. Shown here are a primary tumor (**A**) and a liver metastasis (**B**). After disruption of both alleles of the CD44 gene metastatic spread is almost completely abrogated while incidence and growth rate of osteosarcomas are unaffected (Weber et al., manuscript in preparation)

The biological activity of metastasis-mediating gene products is extensively regulated by posttranscriptional mechanisms. Collagenases are typically secreted as precursors whose activation requires proteolytic cleavage; collagenase type IV becomes active after cleavage by stromelysin while prostromelysin and interstitial procollagenase are activated by plasmin. Ligands for homing receptors often contain multiple domains. The heparin-binding amino-terminus of thrombospondin stimulates chemotaxis while the carboxy-terminus mediates haptotaxis in an RGD-inhibitable fashion. Comparably, a prerequisite for the interaction of the N-terminal osteopontin domain with integrin receptors is phosphorylation of the cytokine while the C-terminal domain engages variant CD44 by protein-protein interaction. The gene for the homing receptor CD44 contains ten variant exons that can be spliced into the extracellular domain and determine its engagement of various ligands. Differential effects on binding to extracellular matrix and hyaluronate also depend on the glycosylation and sulfation status of CD44. Posttranscriptional modification of function of these molecules may be beneficial in two ways. Activation by mechanisms such as proteolytic cleavage and phosphorylation can be accomplished quickly in stress situations; some of the precursor molecules are widely expressed and can acutely be converted at a site of damage. Also, diversity in structure may encode organ specificity in homing and metastasis formation (a "postal code" of sorts). In clinical diagnosis, tumors that grow in a locally invasive manner but do not form distal metastases, including cases of basalioma, glioblastoma, chondrosarcoma, and myelomonocytic leukemia, are often referred to as semimalignant. The molecular mechanisms of local invasion, however, are distinct from conventional forms of cancer only insofar as their target tissues are identical to the tissues of origin.

In conclusion, the topology of cancer spread is regulated by a set of developmentally nonessential genes that physiologically mediate inflammation, wound healing, and neovascularization. The function of their products is extensively regulated posttranscriptionally. The entity of these genes encodes the repertoire of stress responses which are predominantly executed by macrophages and lymphocytes. Metastasis-associated gene products therefore constitute a unique and essential group of cancer related biomolecules whose functions are distinct from those of growth control or senescence genes.

Regulation and dysregulation of metastasis genes

As in the case of yin and yang, phenomena in biology typically have a counterbalance. This also holds true for the regulation of cell dissemination. While tumor suppressor genes inhibit cell cycle progression and serve as antagonists for oncogenes, the genes that mediate metastatic spread are balanced by metastasis suppressor genes. The derived gene products typically are adhesion molecules that procure cell anchorage and inhibit migration. Expression of L-CAM is inversely correlated with the metastatic potential of various tumor cell lines. Loss of cadherin expression in squamous cell carcinomas of the head and neck, prostate cancer, and cancers of the female reproductive tract is associated with poor differentiation and high invasiveness. E-cadherin can prevent the invasive phenotype in T-lymphoma cells. Proteinases also have their antagonists. Tissue inhibitors of metalloproteinases negatively regulate invasion. Their overexpression reduces metastatic potential whereas antisense RNA enhances the malignant phenotype.

It could be argued that metastasis-associated genes are not, in strict terms, cancer genes because mutations in them have not been linked to the risk of contracting cancer. While it is true that these genes have not yet been observed to be mutated in malignancies as in the case of the classical oncogenes (frequently through point mutations, deletions, frame shifts, or translocations), they are subject to dysregulation. A case in point is the expression of ICAM-1 on melanoma cells, which is an indica-

tor of poor prognosis. Similarly, the homing receptor CD44 is often expressed on cancer cells but not at all on their benign precursors. Alternatively, cancer cells may display splice variants of this receptor which are not detected on their nontransformed counterparts. Therefore aberration of genes for cancer spread occurs frequently at the level of transcription or splicing. Without this dysregulation of gene expression tumors could not become malignant.

Metastasis genes and classical cancer genes: the big picture

Even though uncontrolled growth does not inevitably lead to metastatic spread, consistent patterns of organ preference by cancers of particular tissue origin suggest that there is a necessary connection between mutations of oncogenes or tumor suppressor genes and the expression of genes that mediate tumor dissemination. The molecular basis for this connection is currently largely unknown. Expression of metastasis-specific splice variants of CD44 and the oncogene *ras* are connected in an autocatalytic mode in which *ras* induces promoter activity for CD44 through an AP-1 binding site while transfection of CD44v enhances the expression of *ras*. This mutual induction may contribute to the perpetuation of cell division and spreading which are characteristic of malignancy. Motility-associated cytokines, including type IV collagenases and osteopontin, can also be induced by *ras* and similar relationships may apply for other oncogenes, including v-*mos*, v-*raf*, v-*fes*, and v-*src* [2].

Recent research has identified the genes that underlie the three phenotypic characteristics of cancer and has allowed a distinction between malignant and benign tumors at the molecular level. Only tumors in which the dysregulation of growth is associated with expression of genes whose products mediate dissemination become malignant. This attributes a central role in carcinogenesis to metastasis genes and metastasis suppressor genes. The definition of molecules that are rarely expressed in the healthy adult organism has given rise to the potential emergence of new drug targets. Among them are telomerase, structurally altered oncogene products such as fusion proteins or mutants, and also some of the stress response molecules that mediate metastasis formation. Prominently, blocking the integrin $\alpha_v\beta_3$, which is essential for tumor angiogenesis, has been successful in several experimental systems [3, 4]. Likewise, splice variants of CD44 that mediate dissemination of multiple cancers and are physiologically expressed on immune cells only after antigenic challenge have been targeted in experimental therapy with promising results [5, 6, 7]. Such progress provides the opportunity for a more successful broad attack on the cancer epidemic. As the profile of the killer becomes more refined the prospect for its containment improves.

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References

1. Herrlich P, Zoeller M, Pals ST, Ponta H (1993) CD44 splice variants: metastases meet lymphocytes. Immunol Today 14:395–399
2. Greenberg AH, Egan SE, Wright JA (1989) Oncogenes and metastatic progression. Invasion Metastasis 9:360–378
3. Arap W, Pasqualini R, Rhouslahti E (1998) Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. Science 279:377–380
4. Brooks PC, Montgomery AMP, Rosenfeld M, Reisfeld RA, Hu T, Klier G, Cheresh DA (1994) Integrin $\alpha_v\beta_3$ antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. Cell 79:1157–1164
5. Ge L, Resnick NM, Ernst LK, Salvucci LA, Asman DC, Cooper DL (1995) Gene therapeutic approaches to primary and metastatic brain tumors. II. Ribozyme-mediated suppression of CD44 expression. J Neurooncol 26:251–257
6. Hekele A, Dall P, Moritz D, Wels W, Groner B, Herrlich P, Ponta H (1996) Growth retardation of tumors by adoptive transfer of cytotoxic T lymphocytes reprogrammed by CD44v6-specific scFv:zeta-chimera. Int J Cancer 68:232–238
7. Dall P, Hekele A, Beckmann MW, Bender HG, Herrlich P, Ponta H (1997) Efficient lysis of CD44v7/8-presenting target cells by genetically engineered cytotoxic T-lymphocytes – a model for immunogene therapy of cervical cancer. Gynecol Oncol 66:209–216
8. May AE, Kanse SM, Lund LR, Gisler RH, Imhof BA, Preissner KT (1998) Urokinase receptor (CD87) regulates leukocyte recruitment via beta 2 integrins in vivo. J Exp Med 188:1029–1037
9. Schmits R, Filmus J, Gerwin N, Senaldi G, Kiefer F, Kundig T, Wakeham A, Shahinian A, Catzavelos C, Rak J, Furlonger C, Zakarian A, Simard JJL, Ohashi PS, Paige CJ, Gutierrez-Ramos JC, Mak TW (1997) CD44 regulates hematopoietic progenitor distribution, granuloma formation, and tumorigenicity. Blood 90:2217–2233
10. Xu J, Grewal IS, Geba GP, Flavell RA (1996) Impaired primary T cell responses in L-selectin-deficient mice. J Exp Med 183:589–598
11. Shier P, Otulakowski G, Ngo K, Panakos J, Chourmouzis E, Christjansen L, Lau CY, Fung-Leung WP (1996) Impaired immune responses toward alloantigens and tumor cells but normal thymic selection in mice deficient in the beta2 integrin leukocyte function-associated antigen-1. J Immunol 157:5375–5386
12. Sligh JE Jr, Ballantyne CM, Rich SS, Hawkins HK, Smith CW, Bradley A, Beaudet AL (1993) Inflammatory and immune responses are impaired in mice deficient in intercellular adhesion molecule 1. Proc Natl Acad Sci USA 90:8529–8533
13. Xu H, Gonzalo JA, St Pierre Y, Williams IR, Kupper TS, Cotran RS, Springer TA, Gutierrez-Ramos JC (1994) Leukocytosis and resistance to septic shock in intercellular adhesion molecule 1-deficient mice. J Exp Med 80:95–109
14. Lindberg FP, Bullard DC, Caver TE, Gresham HD, Beaudet AL, Brown EJ (1996) Decreased resistance to bacterial infection and granulocyte defects in IAP-deficient mice. Science 274:795–798
15. Liaw L, Birk DE, Ballas CB, Whittsit JM, Davidson JM, Hogan BLM (1998) Altered wound healing in mice lacking a functional osteopontin gene (spp1). J Clin Invest 101:1468–1478
16. Ashkar S, Weber GF, Panoutsakopoulou V, Sanchirico ME, Janssen M, Zawaideh S, Rittling S, Denhardt DT, Glimcher MJ, Cantor H (2000) The cytokine Eta-1/Opn is an essential molecular bridge to type 1 (cell-mediated) immunity. Science 287:860–864

17. Lawler J, Sunday M, Thibert V, Duquette M, George EL, Rayburn H, Hynes RO (1998) Thrombospondin-1 is required for normal murine pulmonary homeostasis and its absence causes pneumonia. J Clin Invest 101:982–992
18. Kunkel EJ, Ley K (1996) Distinct phenotype of E-selectin-deficient mice. E-selectin is required for slow leukocyte rolling in vivo. Circ Res 79:1196–1204
19. Milstone DS, Fukumura D, Padgett RC, O'Donnell PE, Davis VM, Benavidez OJ, Monsky WL, Melder RJ, Jain RK, Gimbrone MA Jr (1998) Mice lacking E-selectin show normal numbers of rolling leukocytes but reduced leukocyte stable arrest on cytokine-activated microvascular endothelium. Microcirculation 5:153–171
20. Subramaniam M, Saffaripour S, Watson SR, Mayadas TN, Hynes RO, Wagner DD (1995) Reduced recruitment of inflammatory cells in a contact hypersensitivity response in P-selectin-deficient mice. J Exp Med 181:2277–2282
21. Shapiro SD (1997) Mighty mice: transgenic technology "knocks out" questions of matrix metalloproteinase function. Matrix Biol 15:527–533
22. Dunsmore SE, Saarialho-Kere UK, Roby JD, Wilson CL, Matrisian LM, Welgus HG, Parks WC (1998) Matrilysin expression and function in airway epithelium. J Clin Invest 102:1321–1331
23. Wilson CL, Ouellette AJ, Satchell DP, Ayabe T, Lopez-Boado YS, Stratman JL, Hultgren SJ, Matrisian LM, Parks WC (1999) Regulation of intestinal alpha-defensin activation by the metalloproteinase matrilysin in innate host defense. Science 286:113–117
24. Rabbani SA, Xing RH (1998) Role of urokinase (uPA) and its receptor (uPAR) in invasion and metastasis of hormone-dependent malignancies. Int J Oncol 12:911–920
25. Plebani M, Herszenyi L, Carraro P, De Paoli M, Roveroni G, Cardin R, Tulassay Z, Naccarato R, Farinati F (1997) Urokinase-type plasminogen activator receptor in gastric cancer: tissue expression and prognostic role. Clin Exp Metastasis 15:418–425
26. Mohanam S, Sawaya RE, Yamamoto M, Bruner JM, Nicholson GL, Rao JS (1994) Proteolysis and invasiveness of brain tumors: role of urokinase-type plasminogen activator receptor. J Neurooncol 22:153–160
27. Naor D, Sionov RV, Zahalka M, Rochman M, Holzmann B, Ish-Shalom D (1998) Organ-specific requirements for cell adhesion molecules during lymphoma cell dissemination. Curr Top Microbiol Immunol 231:143–166
28. Culp LA, Lin WC, Kleinman NR (1999) Tagged tumor cells reveal regulatory steps during earliest stages of tumor progression and micrometastasis. Histol Histopathol 14:879–886
29. Herrlich P, Pals S, Ponta H (1995) CD44 in colon cancer. Eur J Cancer 31A:1110–1112
30. Herrera-Gayol A, Jothy S (1999) Adhesion proteins in the biology of breast cancer: contribution of CD44. Exp Mol Pathol 66:149–156
31. Jensen GS, Po JL, Huerta P, Shustik C (1995) Circulating B-cells in follicular non-Hodgkin's lymphoma show variant expression of L-selectin epitopes. Curr Top Microbiol Immunol 194:171–177
32. Roossien FF, de Rijk D, Bikker A, Roos E (1989) Involvement of LFA-1 in lymphoma invasion and metastasis demonstrated with LFA-1-deficient mutants. J Cell Biol 108:1979–1985
33. Johnson JP (1994–1995) Identification of molecules associated with the development of metastasis in human malignant melanoma. Invasion Metastasis 14:123–130
34. St-Pierre Y, Aoudjit F, Lalancette M, Potworowski EF (1999) Dissemination of T cell lymphoma to target organs: a post-homing event implicating ICAM-1 and matrix metalloproteinases. Leuk Lymphoma 34:53–61
35. Sun JJ, Zhou XD, Liu YK, Tang ZY, Feng JX, Zhou G, Xue Q, Chen J (1999) Invasion and metastasis of liver cancer: expression of intercellular adhesion molecule 1. J Cancer Res Clin Oncol 125:28–34
36. Mawby WJ, Holmes CH, Anstee DJ, Spring FA, Tanner MJ (1994) Isolation and characterization of CD47 glycoprotein: a multispanning membrane protein which is the same as integrin-associated protein (IAP) and the ovarian tumour marker OA3. Biochem J 304:525–530
37. Sharp JA, Sung V, Slavin J, Thompson EW, Henderson MA (1999) Tumor cells are the source of osteopontin and bone sialoprotein expression in human breast cancer. Lab Invest 79:869–877
38. Kasugai S, Zhang Q, Overall CM, Wrana JL, Butler WT, Sodek J (1991) Differential regulation of the 55 and 44 kDa forms of secreted phosphoprotein 1 (SPP-1, osteopontin) in normal and transformed rat bone cells by osteotropic hormones, growth factors and a tumor promoter. J Bone Miner Res 13:235–250
39. Incardona F, Calvo F, Fauvel-Lafeve F, Legrand Y, Legrand C (1993) Involvement of thrombospondin in the adherence of human breast-adenocarcinoma cells: a possible role in the metastatic process. Int J Cancer 55:471–477
40. Albo D, Berger DH, Tuszynski GP (1998) The effect of thrombospondin-1 and TGF-beta 1 on pancreatic cancer cell invasion. J Surg Res 76:86–90
41. Mayer B, Spatz H, Funke I, Johnson JP, Schildberg FW (1998) De novo expression of the cell adhesion molecule E-selectin on gastric cancer endothelium. Langenbecks Arch Surg 383:81–86
42. Zhang GJ, Adachi I (1999) Serum levels of soluble intercellular adhesion molecule-1 and E-selectin in metastatic breast carcinoma: correlations with clinicopathological features and prognosis. Int J Oncol 14:71–77
43. Liu CM, Sheen TS, Ko JY, Shun CT (1999) Circulating intercellular adhesion molecule 1 (ICAM-1), E-selectin and vascular cell adhesion molecule 1 (VCAM-1) in head and neck cancer. Br J Cancer 79:360–362
44. Fox SB, Turner GD, Gatter KC, Harris AL (1995) The increased expression of adhesion molecules ICAM-3, E- and P-selectins on breast cancer endothelium. J Pathol 177:369–376
45. Mannori G, Crottet P, Cecconi O, Hanasaki K, Aruffo A, Nelson RM, Varki A, Bevilacqua MP (1995) Differential colon cancer cell adhesion to E-, P-, and L-selectin: role of mucin-type glycoproteins. Cancer Res 55:4425–4431
46. Engel G, Heselmeyer K, Auer G, Backdahl M, Eriksson E, Linder S (1994) Correlation between stromelysin-3 mRNA level and outcome of human breast cancer. Int J Cancer 58:830–835
47. Adachi Y, Yamamoto H, Itoh F, Hinoda Y, Okada Y, Imai K (1999) Contribution of matrilysin (MMP-7) to the metastatic pathway of human colorectal cancers. Gut 45:252–258
48. Wagner S, Stegen C, Bouterfa H, Huettner C, Kerkau S, Roggendorf W, Roosen K, Tonn JC (1998) Expression of matrix metalloproteinases in human glioma cell lines in the presence of IL-10. J Neurooncol 40:113–122

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Molecular mechanisms of tumor dissemination in primary and metastatic brain cancers

Georg F. Weber[1,2*] and Samy Ashkar[3]

[1]Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, Boston, MA, USA; [2]Department of Medicine, Harvard Medical School, Boston, MA, USA; and [3]Laboratory for Skeletal Disorders and Rehabilitation, Children's Hospital, Department of Orthopedic Surgery, Harvard Medical School, Boston, MA, USA

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ABSTRACT: Cancer is characterized by dysregulated growth control, overcoming of replicative senescence, and metastasis formation. Tumor dissemination distinguishes malignant from benign neoplasms and is mediated by homing receptors, their ligands, and proteinases. The homing receptor CD44 is frequently expressed on primary brain tumors and brain metastases. Its engagement by osteopontin physiologically induces macrophage chemotaxis, a mechanism that may be utilized by metastatic brain tumors in the process of dissemination. In host defense, osteopontin and its receptors, CD44 and integrin $\alpha_V\beta_3$, play key roles in mediating delayed type hypersensitivity responses by activating macrophages to induce Th1 cytokines while inhibiting Th2 cytokines. Other metastasis associated gene products similarly contribute to host defenses. Hence, cancer spread is regulated by a set of developmentally non-essential genes which physiologically mediate stress responses, inflammation, wound healing, and neovascularization. Function of the relevant gene products is extensively modified post-transcriptionally and their dysregulation in cancer occurs on the levels of expression and splicing. Consistent patterns of organ preference by malignancies of particular tissue origin suggest a necessary connection between loss of growth control and senescence genes and expression of genes mediating the dissemination of tumor cells. © 2000 Elsevier Science Inc.

KEY WORDS: Invasion, Homing receptors, Cytokines, Proteinases, Stress response.

MOLECULAR CHARACTERISTICS OF CANCER

The most prominent feature of malignancy is dysregulated cell cycle progression. Division of cancer cells leads to formation of more cancer cells indicating that the characteristics of transformation originate in genetic changes. The underlying defects causing uncontrolled proliferation are gain of function mutations in oncogenes or loss of function mutations in tumor suppressor genes. However, most somatic cells, with few exceptions such as stem cells, die after a finite number of cell divisions, a phenomenon described as senescence. Replicative senescence begins after fertilization and is genetically dominantly controlled. For cancer to occur, there must be a loss of function in senescence genes or a gain of function in telomerase to give rise to a largely unlimited number of cell divisions. Finally, cancer is distinguished from benign tumors by its faculty to generate metastases. In contrast to earlier models, metastasis formation is a process of active cell migration and invasion rather than the passive dyslocation of cells in the blood or lymph flow. Whether a neoplasm metastasizes and to which target organs is determined by motility associated molecules expressed by the tumor cells.

INVASIVENESS OF BRAIN TUMORS

The brain is unique as a target organ for metastatic growth because it is surrounded by the blood—brain barrier and it lacks lymphatic drainage. Nevertheless, certain malignancies display a preference for dissemination to the central nervous system (CNS). Brain metastases from colon and breast cancers are often single, whereas melanoma and lung cancer have a greater tendency to produce multiple colonies. At autopsy, up to 80% of melanoma patients have CNS lesions [20]. Invasion of brain cancer cells typically proceeds along anatomic structures that are rich in extracellular matrix proteins, including basement membranes of blood vessels and the glial limitans externa [4] and has been attributed to specific motility-associated receptors, their ligands and proteinases [6] (Table 1). Specifically, the homing receptor CD44 is frequently expressed on primary brain tumors and brain metastases [10,12,15]. Its ligand osteopontin has also been described to be secreted by malignant gliomas [5,16,22].

THE PHYSIOLOGIC ROLES OF METASTASIS GENES

To understand the process of metastasis formation we have studied the physiologic importance of the relevant gene products. We have investigated the cytokine osteopontin and its receptor CD44 [25–27]. The engagement of CD44 by osteopontin induces macrophage chemotaxis, a process that may be utilized by metastatic brain tumors in the process of dissemination [26]. Gene-targeted mice deficient in osteopontin or CD44 are fertile and developmentally normal, a trait that is shared by other knockouts for genes that are believed to be important in metastatic spread. Several observations implied that osteopontin may act as a stress

* Address for correspondence: Dr. Georg F. Weber, New England Medical Center, 750 Washington Street, NEMC #824, Boston, MA 02111, USA. Fax: +1-(617)-636-1766; E-mail: GWeber@lifespan.org

TABLE 1

METASTASIS-MEDIATING MOLECULES IN BRAIN TUMORS

Tumor	Cytokines	Receptors	Proteinases
Primary brain tumors			
Glioblastoma	Urokinase plasminogen activator, interleukin-8, osteopontin	CD44	Gelatinase-B, active Gelatinase-A, Cathepsin L
Astrocytoma	Hepatocyte growth factor/scatter factor, interleukin-8	c-Met	MT1-MMP, MT2-MMP
Medulloblastoma		polysialylated NCAM	
Metastatic brain tumors			
Melanoma		Neurotrophin receptor	Heparanase
Lung cancer	Urokinase plasminogen activator		
Breast cancer	Urokinase plasminogen activator	Interleukin-6 receptor, CD44	
Prostate cancer		Insulin-like growth factor receptor	
Renal cancer		Interleukin-6 receptor	

Specific receptors, ligands (migration inducing cytokines), and proteinases have been associated with the invasive behavior of individual primary and metastatic brain tumors. MMP, matrix metalloproteinase.

response gene: (1) the osteopontin promoter contains an acute phase responsive element [9] and a phorbol ester responsive element to which the redox sensitive transcription factors Jun and Fos may bind [13], (2) osteopontin expression by T-lymphocytes, macrophages, and osteoclasts does not occur at rest but is activation dependent and is associated with host resistance [13], (3) osteopontin exerts anti-oxidant effects and prevents cell damage in response to a large number of noxious influences [23], (4) the osteopontin gene knockout results in defective wound healing [11]. In host defense, CD44 and its ligand osteopontin play a key role in mediating delayed type hypersensitivity responses by skewing the pattern of cytokines secreted from macrophages to favor the in-

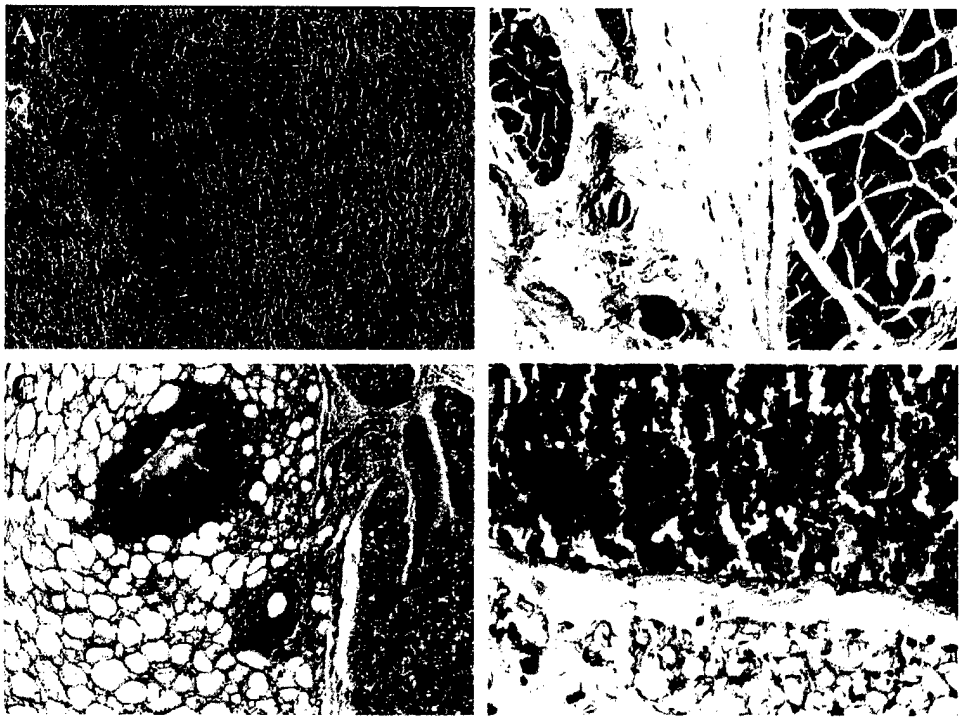

FIG. 1. Absence of a delayed type hypersensitivity response in mice lacking the osteopontin gene. 250 μg polyvinyl pyrrolidone (PVP) in 500 μl phosphate-buffered saline (PBS) was injected subcutaneously into the hind limb of C57BL/6 wildtype mice or gene targeted C57BL/6 $OPN^{-/-}$ mice which do not express the osteopontin gene product. After 5 days, histologic analysis of the injection site was performed; 5-μm serial sections were stained with hematoxilin-eosin stain. (A) Injection of PVP into C57BL/6; (B) injection of PBS (vehicle control) into C57BL/6; (C) injection of PVP into C57BL/6 $OPN^{-/-}$ and (D) injection of PVP in conjunction with 10 μg purified osteopontin into C57BL/6 $OPN^{-/-}$. Original magnification 200×.

TABLE 2
GENES ASSOCIATED WITH MALIGNANCIES

Genes	Function	Examples
Oncogenes	Growth factors	EGF, PDGF
	Growth factor receptors	HER-2, erb-B
	Signal transduction molecules associated with growth factor receptors	Akt, Abl, Ras
Tumor suppressor genes	Receptors	DCC,PTC
	Signal transduction molecules	p53, Rb,APC
Senescence genes	Cell cycle regulators	p53,Rb,p21,Fos
Senescence suppressor genes	Regulators of telomere length	Telomerase
Metastasis genes	Homing receptors and their ligands	CD44, selectins, osteopontin
	Proteinases	MMPs
Metastasis suppressor genes	Adhesion receptors	cadherins, L-CAM, KAI1
	Proteinase inhibitors	TIMPs
Mutator genes	Mismatch repair	MSH, PMS
	Base excission repair	Uracil DNA glycosylase
	Nucleotide excission repair	ERCC
	Repair of double strand breaks	XRCC,RAD50,NSB1

The classical cancer genes (oncogones and tumor suppressor genes) control cell replication. For cancer to occur, additional functions need to be dysregulated: genes that cause cellular senescence have to be inactivated and expression of gene products that mediate metastasis formation is essential. For cell cycle progression and cell dissemination alike, there is a physiologic balance that may be disturbed by excessive activity of promoters or by diminished function of suppressors. Defects in mutator genes give rise to alterations in other cancer-associated genes putting mutator genes into the position of predisposing factors rather than direct contributors to the malignant phenotype.

duction of cellular immunity and to suppress humoral immunity. The interaction of osteopontin with its integrin receptor $a_V\beta_3$ on macrophages stimulates the production of Th1 cytokines while engagement of CD44 by osteopontin concomitantly inhibits the secretion of Th2 cytokines [2]. A classical model of delayed type hypersensitivity is granuloma formation. Foreign body granulomas can be induced by subcutaneous injection of polyvinyl pyrrolidone. After 5 days, control mice display pronounced influx of macrophages and a strong local immune response. In contrast, mice lacking the osteopontin gene due to targeted mutation barely show any immunological reaction to the injection (Fig. 1). In contrast, mice lacking the CD44 gene display excessive granuloma formation following challenge [17] which may reflect combined Th1 and Th2 immunity after engagement of integrin receptors by osteopontin in the absence of ligation of CD44.

Preliminary experiments have suggested that $CD4^+$ T-cells secrete the osteopontin that induces macrophages to selectively promote delayed type immune responses. The observation that macrophages may themselves produce osteopontin after stimulation with lipopolysaccharide raises questions regarding its potential relevance to this process. Macrophage-derived osteopontin is competent for inducing chemotaxis but not delayed type hypersensitivity which may reflect structural differences from the T-cell secreted molecule. In fact, macrophage osteopontin has lost part of its sequence by alternative splicing [Ashkar and Weber, unpublished observations] which could lead to efficient engagement of CD44 with ensuing chemotaxis but to impaired ligation of integrin receptors. Malignant cells often secrete a form of osteopontin that resembles the macrophage-derived protein in that it may be hypophosphorylated or a splice variant that has a deletion in its N-terminal (integrin binding) portion [8] and this molecule may contribute to metastatic spread [25] by inducing tumor cell migration. Concomitantly, tumor-derived modified osteopontin may ligate CD44 on macrophages without engagement of its integrin receptors [19]. This leads to suppression of Th2 cytokines while Th1 cytokines cannot be efficiently secreted since other physiologic inducers of Th1 cytokines are substantially less potent. This form of osteopontin action may represent a mechanism of immune evasion.

Tumor dissemination depends on neovascularization. Physiologically, blood vessel formation may be initiated in two settings. The modeling of the cardiovascular system is largely restricted to early development, while in the healthy adult organism, angiogenesis is a rare occurrence that arises predominantly in healing after tissue damage. Morphogenic and stress induced blood vessel generation are mediated by distinct sets of genes. Several pieces of evidence imply a role for osteopontin and its receptors in the latter form of neovascularization. A splice variant of CD44 is involved in endothelial cell proliferation, migration, and angiogenesis [7, 21]. The integrin $a_V\beta_3$ is of particular importance in angiogenesis due to its selective expression on growing blood vessels. Antagonists of integrin $a_V\beta_3$ promote tumor regression by inhibiting neovascularization [1,3] and angiogenesis induced by bFGF or by TNFα is also inhibitable by a monoclonal antibody to the integrin $a_V\beta_3$. Coordinate expression of β_3-integrins and osteopontin by regenerating endothelial cells [11] and during in vitro blood vessel formation [14] stimulates migration through cooperative mechanisms involving activation of integrin $a_V\beta_3$ ligation by thrombin cleavage of osteopontin [18].

CONCLUSIONS

We conclude, based on our own observations in conjunction with data from the literature, that the topology of cancer spread is regulated by a set of developmentally non-essential genes which physiologically mediate stress responses, inflammation, wound healing, and neovascularization and are normally expressed by activated lymphocytes and macrophages [24]. Function of the relevant gene products is extensively modified post-transcriptionally which allows for quick activation in stress situations and may encode organ specificity. This code for targets in the homing process may cause dissemination to distant organs, such as brain metastases in melanoma or lung cancer, or it may lead to locally invasive growth as is the case in malignant glioma or in chondrosarcoma. In both scenarios, locally destructive growth by malignant glioma and brain metastases from distant primary tumors, the mechanism of invasion is determined by engagement of molecules that are physiologically used by macrophages and lymphocytes to enter the central nervous system in the context of host defenses, including infection, inflammation, or ischemia.

Indicative of basic mechanisms of homeostasis in human biology, all groups of genes involved in malignancies consist of promoting and suppressing components. Loss of function in one group or gain of function in the counterbalancing group may each affect the balance of forces and constitute a predisposing factor for malignant growth. Thus, mutations that enhance the function of oncogenes and mutations that inhibit the function of tumor suppressor genes equally pose a risk for uncontrolled growth of the affected cells and similar relationships hold for senescence genes and metastasis genes and their respective suppressors (Table 2). Furthermore, consistent patterns of organ preference by cancers of particular tissue origin suggest that there is a necessary connection among dysregulated cell cycle control genes (gain of function of oncogenes or loss of function of tumor suppressor genes), suppression of senescence genes, and expression of genes mediating the dissemination of tumor cells. Today, the molecular basis for this connection is largely unknown.

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REFERENCES

1. Arap, W.; Pasqualini, R.; Rhouslahti, E. Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. Science 279: 377–380; 1998.
2. Ashkar, S.; Weber, G. F.; Panoutsakopoulou, V.; Sanchirico, M. E.; Janssen, M.; Zawaideh, S.; Rittling, S.; Denhardt, D. T.; Glimcher, M. J.; Cantor, H. The cytokine Eta-1/Opn is an essential molecular bridge to type 1 (cell-mediated) immunity. Science 287:860–864; 2000.
3. Brooks, P. C.; Montgomery, A. M. P.; Rosenfeld, M.; Reisfeld, R. A.; Hu, T.; Klier, G.; Cheresh, D. A. Integrin $\alpha_v\beta_3$ antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. Cell 79:1157–1164; 1994.
4. Giese, A.; Westphal, M. Glioma invasion in the central nervous system. Neurosurgery 39:235–252; 1996.
5. Gladson, C. L. The extracellular matrix of gliomas: Modulation of cell function. J. Neuropathol. Exp. Neurol 58:1029–1040; 1999.
6. Goldbrunner, R. H.; Bernstein, J. J.; Tonn, J.-C. Cell-extracellular matrix interaction in glioma invasion. Acta Neurochir. 141:295–305; 1999.
7. Henke, C. A.; Roongta, U.; Mickelson, D. J.; Knutson, J. R.; McCarthy, J. B. CD44-related chondroitin sulfate proteoglycan, a cell surface receptor implicated with tumor cell invasion, mediates endothelial cell migration on fibrinogen and invasion into a fibrin matrix. J. Clin. Invest. 97:2541–2552; 1996.
8. Kiefer, M. C.; Bauer, D. M.; Barr, P. J. The cDNA and derived amino acid sequence for human osteopontin. Nucl. Acid Res. 17:3306; 1989.
9. Kimbro, K. S.; Saavedra, R. A. The puerap motif in the promoter of the mouse osteopontin gene. Ann. N.Y. Acad. Sci. 760:319–320; 1995.
10. Kuppner, M. C.; van Meir, E.; Gauthier, T.; Hamou, M. F.; de Tribolet, N. Differential expression of the CD44 molecule in human brain tumours. Int. J. Cancer 50:572–577; 1992.
11. Liaw, L.; Birk, D. E.; Ballas, C. B.; Whittsit, J. M.; Davidson, J. M.; Hogan, B. L. M. Altered wound healing in mice lacking a functional osteopontin gene (*spp1*). J. Clin. Invest. 101:1468–1478; 1998.
12. Nagasaka, S.; Tanabe, K. K.; Bruner, J. M.; Saya, H.; Sawaya, R. E.; Morrison, R. S. Alternative splicing of the hyaluronic acid receptor CD44 in the normal human brain and in brain tumors. J. Neurosurg. 82:858–863; 1995.
13. Patarca, R.; Saavedra, R. A.; Cantor, H. Molecular and cellular basis of genetic resistance to bacterial infection: The role of the early T-lymphocyte activation-1/Osteopontin gene. Crit. Rev. Immunol. 13: 225–246; 1993.
14. Prols, F.; Loser, B.; Marx, M. Differential expression of osteopontin, PC4, and CEC5, a novel mRNA species, during in vitro angiogenesis. Exp. Cell. Res. 239:1–10; 1998.
15. Radotra, B.; McCormick, D.; Crockard, A. CD44 plays a role in adhesive interactions between glioma cells and extracellular matrix components. Neuropathol. Appl. Neurobiol. 20:399–405; 1994.
16. Saitoh, Y.; Kuratsu, J.; Takeshima, H.; Yamamoto, S.; Ushio, Y. Expression of osteopontin in human glioma. Its correlation with the malignancy. Lab. Invest. 72:55–63; 1995.
17. Schmits, R.; Filmus, J.; Gerwin, N.; Senaldi, G.; Kiefer, F.; Kundig, T.; Wakeham, A.; Shahinian, A.; Catzavelos, C.; Rak, J.; Furlonger, C.; Zakarian, A.; Simard, J. J.; Ohashi, P. S.; Paige, C. J.; Gutierrez-Ramos, J. C.; Mak, T. W. CD44 regulates hematopoietic progenitor distribution, granuloma formation, and tumorigenicity. Blood 90:2217–2233; 1997.
18. Senger, D. R.; Ledbetter, S. R.; Claffey, K. P.; Papadopoulos-Sergiou, A.; Peruzzi, C. A.; Detmar, M. Stimulation of endothelial cell migration by vascular permeability factor/vascular endothelial growth factor through cooperative mechanisms involving the alphavbeta3 integrin, osteopontin, and thrombin. Am. J. Pathol. 149:293–305; 1996.
19. Shanmugam, V.; Chackalaparampil, I.; Kundu, G. C.; Mukherjee, A. B.; Mukherjee, B. B. Altered sialylation of osteopontin prevents its receptor-mediated binding on the surface of oncogenically transformed tsB77 cells. Biochemistry 13:5729–5738; 1997.
20. Sugarbaker, E. V. Patterns of metastases in human malignancies. Cancer Biol. Rev. 2:235–278; 1981.
21. Trochon, V.; Mabilat, C.; Bertrand, P.; Legrand, Y.; Smadja-Joffe, F.; Soria, C.; Delpech, B.; Lu, H. Evidence of involvement of CD44 in endothelial cell proliferation, migration and angiogenesis in vitro. Int. J. Cancer 66:664–668; 1996.
22. Tucker, M. A.; Chang, P. L.; Prince, C. W.; Gillespie, G. Y.; Mapstone, T. B. TPA-mediated regulation of osteopontin in human malignant glioma cells. Anticancer Res. 19:807–812; 1998.
23. Weber, G. F.; Adler, B.; Ashkar, S. Osteopontin in oxidative stress responses. In: Ruffolo, R. R. Jr.; Feuerstein, G. Z.; Hunter, A. J.; Poste, G.; Metcalf, B. W., eds. Inflammatory cells and mediators in CNS diseases. Amsterdam: Harwood Academic Publishers; 1999:97–112.
24. Weber, G. F.; Ashkar, S. Stress response genes—The genes that make cancer metastasize. J. Mol. Med. 78:404–408; 2000.
25. Weber, G. F.; Ashkar, S.; Cantor, H. Interaction between CD44 and osteopontin as a potential basis for metastasis formation. Proc. Assoc. Am. Physicians 109:1–9; 1997.
26. Weber, G. F.; Ashkar, S.; Glimcher, M. J.; Cantor, H. Receptor-ligand interaction between CD44 and osteopontin (Eta-1). Science 271:509–512; 1996.
27. Weber, G. F.; Cantor, H. The immunology of Eta-1/osteopontin. Cytokine Growth Factor Rev. 7:241–248; 1996.

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Costimulation by Extracellular Matrix Proteins Determines the Response to TCR Ligation

Barbara Adler,*,†,[1] Samy Ashkar,‡,§ Harvey Cantor,*,† and Georg F. Weber*,¶,[2]

*Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute; ¶Department of Medicine, †Department of Pathology, §Department of Orthopedic Surgery, Harvard Medical School; and ‡Laboratory for Skeletal Disorders and Rehabilitation, Department of Orthopedic Surgery, Children's Hospital, Boston, Massachusetts 02115

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Although ligation of the T-cell antigen receptor (TCR) is central to the responsiveness and antigen specificity of T-cells, it is insufficient to elicit a response. To determine whether the need for costimulation reflects inadequate strength of signal transduction through the TCR or an absolute block of signaling in the absence of a coligand, we studied T-cell activation under serum-free conditions eliminating costimulation by various extracellular matrix proteins which otherwise have an omnipresent and frequently overlooked effect. Engagement of the TCR leads to induction of Fas, but not to measurable IL-2 secretion or apoptosis. Those activation parameters are induced by costimulation through integrin $\alpha_V\beta_3$. Furthermore, T-cell survival or elimination is determined by the type of ligand binding to this coreceptor with vitronectin, fibronectin, and fibrinogen efficiently inducing apoptosis and IL-2 production while osteopontin and entactin mediate IL-2 secretion comparably without causing programmed cell death. Consistent with the cytokine properties of these ligands, differential costimulation depends on their presentation in soluble rather than immobilized form. The determination of elimination versus survival of activated T-cells by coligation of β_3-integrins may have bearing on the fundamental postthymic mechanisms that shape the T-cell repertoire. © 2001 Academic Press

Key Words: **T-cell; costimulation; integrin; extracellular matrix protein; vitronectin; osteopontin; apoptosis; interleukin-2.**

INTRODUCTION

Cognate ligation of the T-cell antigen receptor (TCR) is an essential prerequisite for specific T-lymphocyte-dependent immunity; however, a contributing role of costimulatory molecules to T-cell responsiveness has been increasingly recognized. Two hypotheses can be put forward regarding the effect of costimulation: 1) The conventional strength of signal paradigm proposes that signaling through the T-cell antigen receptor can be enhanced by costimuli, but that strong enough engagement of the T-cell antigen receptor would be transduced to the nucleus (1). Ligation of the T-cell antigen receptor by an antigen with high affinity or at a high dose can mediate independence of costimulation (2). This model is weakened by observations that late signals and secondary signals may have costimulatory characteristics even though they have little to do with the strength of the signal transduction through the TCR. Coligation of CD4 converges with the TCR-associated signal very proximally while the convergence of CD28 signal transduction with the TCR is more distal. A prerequisite for Fas-dependent apoptosis is the expression of FasL following TCR ligation so that this mode of programmed cell death constitutes a delayed, secondary signal. 2) Alternatively, costimulation may overcome blocks in signal transduction, which would otherwise prevent efficient T-cell activation. Costimulation through CD28 may vary according to expression levels of B7 on antigen-presenting cells or according to presentation of B7-1 or B7-2 and can result in marked differences in the type of T-cell responses induced (3, 4). Nevertheless, the existence of costimulation-dependent checkpoints in T-cell signal transduction has not been demonstrated.

Effective T-cell costimulation can be transduced through integrins and may occur in the absence of CD28 (5), implying an important role for integrin-associated signals in T-cell activation. Some studies have found integrins to only facilitate T-cell activation by lowering an activation threshold without qualitatively changing the T-cell response (6, 7). It was suggested that CD11a/CD18 is important in initial contact between T-cells and antigen-presenting cells and in strengthening this interaction. In other investigations,

[1] Current address: Haematologikum der GSF, Institut für klinische Molekularbiologie, Marchioninistrasse 25, 81377 München, Germany.

[2] To whom correspondence should be addressed at New England Medical Center, Tufts University Medical School, 750 Washington Street, NEMC No. 824, Boston, MA 02111. Fax: (617) 636-1766. E-mail: gweber@lifespan.org.

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however, integrin-dependent costimulation was characterized as essential for the prevention of programmed cell death (8, 9) or for overcoming unresponsiveness (10, 11). Divergent observations and interpretations of integrin contributions to T-cell activation may have been caused by failure to consider differential effects by various ligands on T-cell costimulation through integrins.

In the past, costimulation experiments have typically been performed in serum-containing medium so that ligation of integrin receptors by serum components has always been present and has affected the assessment of T-cell activation. Furthermore, costimulation in T-cell activation has been investigated mostly with regard to proliferation and interleukin-2 secretion (12). The contribution of accessory signals to other readouts of T-cell stimulation, including apoptosis, is insufficiently understood. Specifically, it is unknown whether there is a segregation of functions among specific costimuli, some of which lead to proliferation and cytokine secretion and others to programmed cell death. We addressed these questions by analyzing T-cell responses to ligation of CD3 in conditioned medium that avoids inadvertent addition of coligands contained in fetal calf serum.

MATERIALS AND METHODS

Cells. The AF3.G7 hybridoma was generated by fusing cow insulin immune C57BL/6 lymph node cells with the BW5147 thymoma line (13). It was shown by flow cytometry to be positive for CD3, CD4, CD44, and CD51/CD61 (integrin $\alpha_V\beta_3$). The cells were maintained in DMEM (Sigma, St.Louis, MO) supplemented with 10% fetal bovine serum (FBS), L-glutamine, Hepes, and 50 μM 2-mercaptoethanol. To eliminate cell-line-specific nonphysiologic effects, a second hybridoma, BC.C10, that had been derived from fusion of BC.4 cells with BW5147, bears H-2^b, and expresses TCR $V_\beta 3$, was used for independent confirmation of the results in a second cell line. S49.33 (ATCC cell line TIB 35) and AKR1.1 (ATCC cell line TIB 232) are $CD3^+$ T-cell lines negative for integrin $\alpha_V\beta_3$.

Peripheral $CD4^+$ T-cells were obtained from lymph nodes of C57Bl/6 mice by negative selection with Dynabeads coated with anti-CD8 and anti-CD45R (B220) antibodies. The cells were stimulated at 1×10^6/ml with 2 μg/ml concanavalin A for 3 days and expanded in culture at a density of 0.5×10^6 cells/ml with 10 U/ml IL-2 for 4 consecutive days. After confirmation of high-level expression of the relevant surface markers CD3, CD4, integrin α_V, and integrin β_3, the cells were used for stimulation experiments.

Antibody crosslinking and cell activation. Anti-mouse CD3 antibody 145.2C11 (Pharmingen, San Diego, CA) was plated at 0.5 μg/well by diluting in 0.5 ml PBS, pH 8.5, and incubation in 24-well plates for 24 h at 4°C. The plates were washed three times with PBS. Where indicated, osteopontin was coimmobilized with anti-CD3. At the concentrations used, osteopontin and anti-CD3 are unlikely to impair one another's plating efficiencies. T-cells were then added at a concentration of 2.5×10^5 cells/well in a volume of 0.5 ml of serum-free medium, either AIM-V medium (Gibco/BRL) or DMEM in which serum had been replaced with 5 μg/ml insulin and 5 μg/ml transferrin. Extracellular matrix proteins, including murine or bovine vitronectin (Gibco/BRL), osteosarcoma-derived osteopontin variant A from the osteosarcoma cell line K7 (14), fibronectin (Gibco/BRL), fibrinogen (Sigma), or entactin fragment (Sigma), were added at the specified concentrations. Where indicated, fetal bovine serum was present at 5%. Blocking antibodies used were anti-integrin β_3 (2C9.G2), anti-integrin β_1 (9EG7, Ha2/5), anti-integrin β_2 (GAME-46), and anti-integrin β_7 (FIB27), all obtained from Pharmingen. Also used were antibodies anti-Pgp1 (clone IM7, Pharmingen), KM81 (purified from ATCC clone TIB241), and anti-Ly24A.2 (Biosource). For competition studies, cells were preincubated in a small volume with the relevant antibodies for 15 min at 4°C and plated afterward. Supernatants or cells were harvested after 16 h for determination of IL-2 levels by ELISA and apoptosis by flow cytometry, respectively.

Analysis of surface markers. Expression of cell surface markers was determined by flow cytometry with fluorescently labeled antibodies. T-cell hybridomas were routinely stained for CD3, CD4, CD8, and controls after thawing and after several weeks in culture. For analysis of Fas expression, PE-anti-Fas antibody was used with PE-anti-CD8 and PE-anti-CD3 as negative and positive controls, respectively. All antibodies were obtained from Pharmingen.

IL-2 ELISA. Levels of IL-2 in cell culture supernatants were measured following standard protocols (Pharmingen). Cell culture supernatants were analyzed at 1:10 or 1:20 dilution and color development was achieved with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) plus hydrogen peroxide.

Apoptosis assay. T-cell apoptosis was determined by staining with propidium iodide and measurement of a subdiploid peak (15). Cells were washed in PBS/5 mM EDTA and porated by adding 100% ethanol to a final concentration of 50% while vortexing. After a 30-min incubation at room temperature cells were pelleted, resuspended in PBS/5 mM EDTA with 40 μg/mL RNAse A (Sigma), and incubated for consecutive 30 min, followed by addition of an equal volume of 100 μg/ml propidium iodide for analysis by flow cytometry.

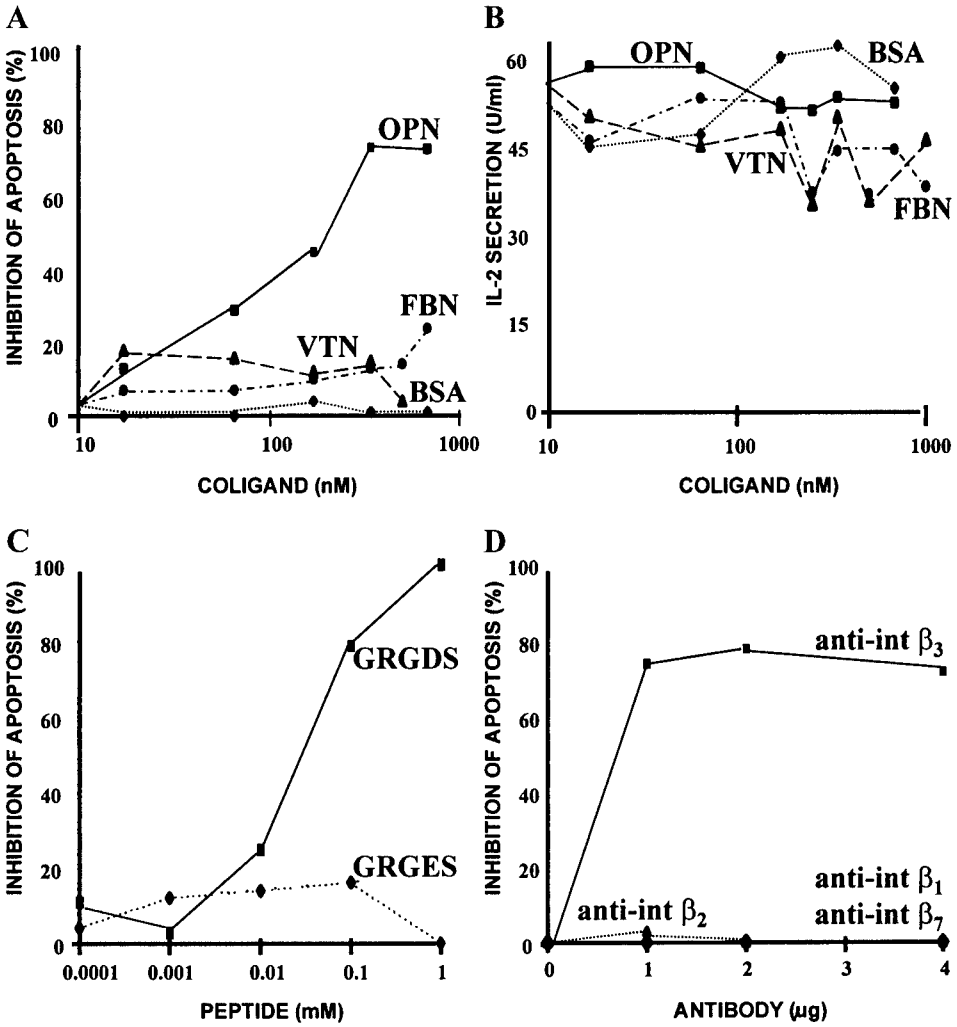

FIG. 1. Modulation of T-cell stimulation by extracellular matrix proteins. AF3.G7 cells, grown in DMEM containing 5% FBS, were stimulated with 0.5 μg/well plated anti-CD3 in 24-well plates for 16 h before analysis of apoptosis and IL-2 secretion. Extracellular matrix proteins (A,B) or blocking agents for integrin receptors (C,D) were present in soluble form at the indicated concentrations. (A) Osteopontin inhibits apoptosis in a dose-dependent manner, whereas vitronectin and fibronectin have no effect. Control levels of apoptosis induced by plated anti-CD3 antibody after 24 h are 55%. (B) Osteopontin, vitronectin, and fibronectin do not alter anti-CD3-induced levels of IL-2 secretion (U/ml). (C) GRGDS peptide, but not GRGES control peptide, inhibits apoptosis dose-dependently. (D) Addition of a blocking anti-integrin β_3 antibody, but not control immunoglobulin (not shown), inhibits apoptosis. Even though integrin β_1 is expressed on AF3.G7 cells a specific antibody does not affect activation; this is also the case for antibodies to integrins β_2 and β_7. All agents tested have no effect on apoptosis or IL-2 secretion levels when added to resting cells.

RESULTS

Extracellular matrix proteins provide differential costimuli. The T-cell hybridoma AF3.G7, grown in DMEM with 5% FBS, responds to ligation of the T-cell antigen receptor via plated anti-CD3 antibody with secretion of IL-2 and apoptosis (16). We tested the effects of costimulation by extracellular matrix proteins on activation as judged by these readouts. Unexpectedly, osteopontin inhibited apoptosis in a dose-dependent manner, whereas vitronectin and fibronectin had no effect; none of the ligands tested affected IL-2 secretion (Figs. 1A and 1B). Commonly, these ligands contain RGD motifs that are important for engagement of integrin receptors. When we added GRGDS peptide to the medium to compete with the RGD-mediated binding we found dose-dependent inhibition of apoptosis (Fig. 1C). Furthermore, addition of a blocking anti-integrin β_3 antibody, but not control immunoglobulin, inhibited programmed cell death while antibodies to integrins β_1, β_2, and β_7 did not (Fig. 1D), suggesting that costimuli acting through β_3-integrin receptors were already provided with the medium.

Costimulation through integrin β_3 is essential for T-cell activation. After elimination of serum from the medium, stimulation with plated anti-CD3 does not result in any measurable IL-2 secretion or apoptosis. This is the case even at very high concentrations of anti-CD3 antibody at which the apoptotic response in

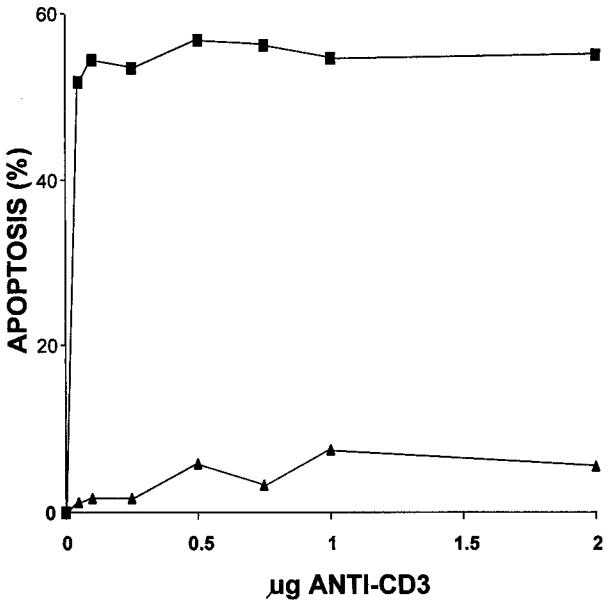

FIG. 2. Titration of plate-bound anti-CD3 antibody. AF3.G7 cells were stimulated with the indicated concentrations of plated anti-CD3 in 24-well plates for 16 h, either in DMEM containing 5% FBS (filled squares) or in serum-free medium (filled triangles), before analysis of apoptosis and IL-2 secretion. Even at high concentrations of anti-CD3 antibody (2 µg/well) there is no substantial induction of apoptosis in serum-free medium, whereas the level of programmed cell death induced in the presence of serum reaches near maximal levels at moderate amounts of anti-CD3 (0.5 µg/well).

serum-containing medium has reached a plateau (Fig. 2). Therefore, increased strength of signaling through the TCR cannot overcome the absence of costimuli.

Addition of individual extracellular matrix proteins, including vitronectin, fibronectin, or fibrinogen, restored IL-2 secretion and apoptosis, whereas addition of osteopontin or entactin was somewhat less effective at recovering IL-2 secretion and did not lead to induction of apoptosis. (Figs. 3A–3D). Similar results were obtained with another T-cell hybridoma (BC.C10, data not shown).

Commonly, the extracellular matrix proteins under study may ligate the integrin receptor $\alpha_V\beta_3$ and indeed a blocking anti-integrin β_3 antibody reversed the effects of all ligands (Figs. 3E and 3F). In contrast, two T-cell lines (S49.33 and AKR1.1) that do not express the candidate receptor integrin $\alpha_V\beta_3$ for these molecules did not respond to plated anti-CD3 with IL-2 secretion or apoptosis, even in serum-containing medium (data not shown). These ligands often display substantial receptor promiscuity. Fibrinogen, fibronectin, and entactin may also ligate β_1-integrins. We asked which integrins contribute to costimulation induced by these extracellular matrix proteins under serum-free conditions. Addition of a blocking anti-integrin β_1 antibody did not reverse their costimulatory effects whereas addition of anti-integrin β_3 antibody mediated complete inhibition (Figs. 3E and 3F). We also tested whether a second receptor for osteopontin, CD44, might be involved. CD44 has been associated with T-cell costimulation (17–19) and specifically with inhibition of T-cell apoptosis (20, 21). Three different antibodies to CD44 (anti-Pgp1, KM81, anti-Ly24A.2) did not affect osteopontin-dependent costimulation (Figs. 3E and 3F), serum-induced costimulation, or the anti-apoptotic effect of osteopontin in complete medium (data not shown). Phosphorylation is necessary for ligation of integrin receptors by osteopontin, and consistent with the above results, we found that bacterially produced recombinant osteopontin, which does not have posttranslational modifications, had no effect on apoptosis (data not shown). This was not due to a lack of recognition of recombinant osteopontin by AF3.G7 cells since this hybridoma has been shown to bind unmodified recombinant osteopontin via its CD44 receptor (22).

Ligands can compete for a receptor and thus modulate the response phenotype. The anti-apoptotic effect in complete medium of osteopontin but not vitronectin suggested competition between vitronectin contained in serum and the added osteopontin ligand. Because vitronectin is a potent costimulus at very low doses, other extracellular matrix proteins contained in serum may contribute less. We tested for competition between osteopontin and vitronectin under serum-free conditions. Increasing concentrations of osteopontin competed with a fixed amount of 68 nM vitronectin, resulting in curves that reflect the combination of competition and osteopontin-induced signal transduction. Dose-dependent inhibition of apoptosis was observed but the IL-2 curve shows increasing levels at higher concentrations of osteopontin (Figs. 3G and 3H). These data corroborate that the two ligands induce distinct response phenotypes after ligation of the same receptor.

Immobilized osteopontin exerts a nonspecific RGD effect. Extracellular matrix molecules may ligate cell surface receptors in soluble form after secretion by immune cells. As part of the intercellular matrix, they may also engage integrins as cross-linked stimuli. We tested whether the presentation of osteopontin in immobilized form impacts its effect as T-cell costimulus (23). Indeed, plate-bound osteopontin inhibited IL-2 secretion and apoptosis after serum-mediated costimulation comparably to GRGDS, whereas equal molar amounts of BSA had no effect. While the RGD sequence in extracellular matrix proteins is necessary for the engagement of integrins, it is not sufficient for specific signal transduction. We have identified phosphorylation of an N-terminal domain of osteopontin as a prerequisite for specific interaction with integrin $\alpha_V\beta_3$ (24). Therefore, inhibition of IL-2 secretion and apoptosis by plated osteopontin is consistent with blocking of integrin receptors through the RGD motif and suggests that immobilization inactivates osteopontin signaling through steric hinderance (Figs. 4A–4C).

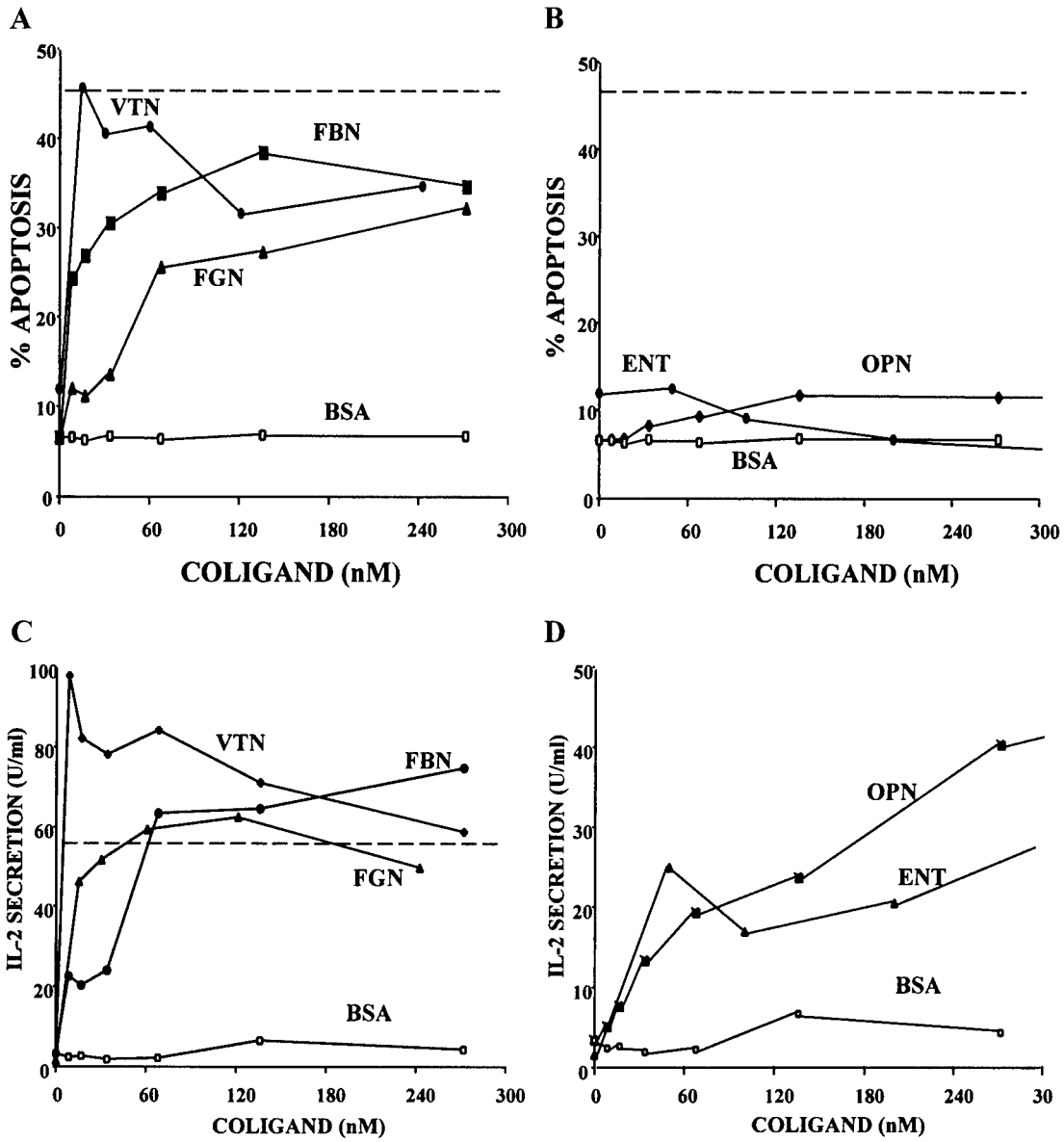

FIG. 3. T-cell costimulation by extracellular matrix proteins in serum-free medium. AF3.G7 cells, grown in serum-free DMEM, were stimulated with 0.5 μg/well plated anti-CD3 in 24-well plates for 16 h before analysis of apoptosis and IL-2 secretion. Extracellular matrix proteins were present in soluble form at the indicated concentrations. (A–D) Addition of vitronectin, fibronectin, or fibrinogen restores IL-2 secretion (C) and apoptosis (A), while addition of osteopontin or entactin restores IL-2 secretion (D), but not apoptosis (B). The horizontal dashed lines indicate the extent of IL-2 secretion or apoptosis stimulated by anti-CD3 in the presence of 5% fetal bovine serum. (E,F) Costimulation by extracellular matrix molecules is reversible by a blocking antibody to integrin β_3. Even though fibrinogen and entactin may also ligate integin β_1 an antibody to this integrin has no effect. Likewise, three distinct antibodies to CD44 do not inhibit osteopontin-dependent costimulation. (G,H) Competition between a fixed amount of 68 nM vitronectin and increasing concentrations of osteopontin for ligation of the coreceptor leads to diminished apoptosis with increasing dose of osteopontin. The curve for IL-2 levels reflects the combined effects of competition with vitronectin binding and intrinsic IL-2 induction by osteopontin.

In the absence of costimulation, the block in signal transduction is incomplete. The absence of IL-2 secretion or apoptosis without costimulation may reflect a complete block of TCR-associated signal transduction. Because the expression of Fas is frequently induced after T-cell activation and predisposes to ensuing apoptosis, we asked whether its induction is dependent on costimulation. While the level of CD3 expression remained constant over time, the levels of Fas increased during 16 h after TCR ligation. Induction of Fas was modestly enhanced by costimuli (fourfold) but was present after ligation of CD3 in serum-free medium (threefold) (Figs. 5A and 5B). The costimulation-independent induction could not be reversed by the addition of anti-integrin β_3 antibody (data not shown), indicating that the effect is mediated through the TCR

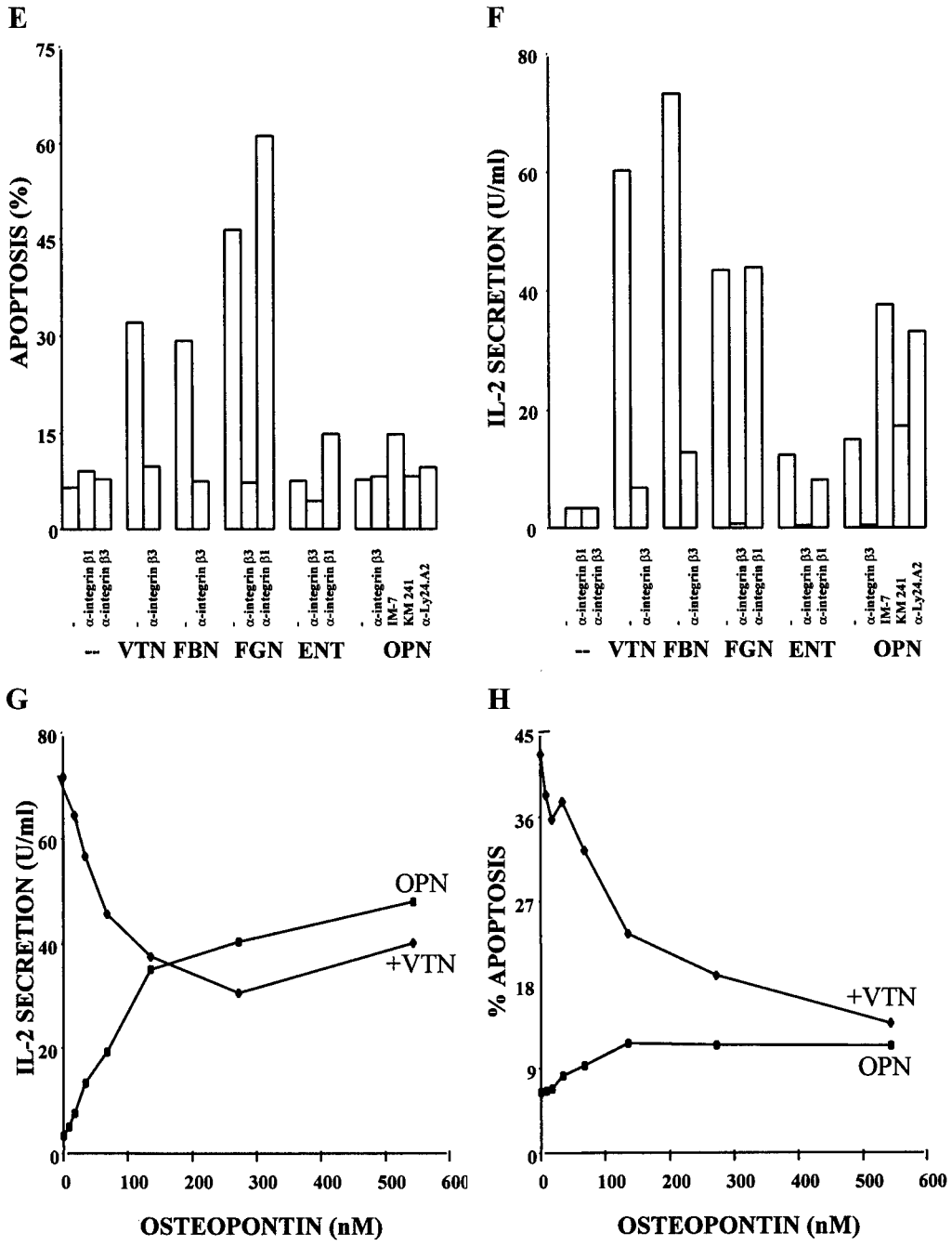

FIG. 3—*Continued*

and is not due to incomplete removal of serum components.

Osteopontin and vitronectin provide differential costimulation in peripheral CD4⁺ cells. Naive lymph-node-derived T-cells express low levels of the integrin $\alpha_V\beta_3$ (25), but receptor expression is induced consecutive to activation. After being primed with concanavalin A followed by IL-2, lymph-node-derived $CD4^+$ cells were stimulated effectively by vitronectin or osteopontin to produce IL-2 and differentially underwent apoptosis in response to vitronectin but not osteopontin (Figs. 6A and 6B).

DISCUSSION

Previous experiments supporting the notion that signaling through the T-cell antigen receptor can be enhanced by costimuli but that a strong enough primary stimulus would be transduced to the nucleus may have been misleading, since they were frequently performed

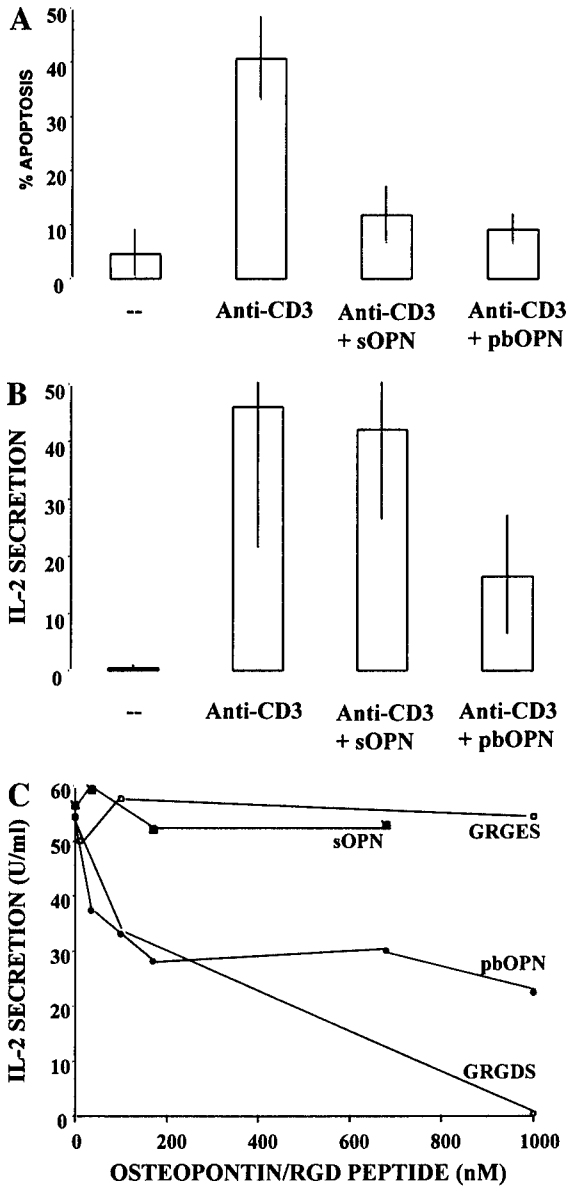

FIG. 4. Immobilized osteopontin exerts an RGD effect. AF3.G7 cells were incubated in DMEM containing 5% FBS with plated anti-CD3. The indicated coligands were presented at the indicated concentrations in soluble form or after immobilization together with anti-CD3 (this did not diminish plating efficiency). (A,B) While soluble osteopontin inhibits apoptosis without affecting IL-2 secretion in serum containing medium, plated osteopontin inhibits both readouts. (C) Plated osteopontin and GRGDS inhibit IL-2 secretion dose-dependently whereas soluble osteopontin and GRGES do not.

in serum-containing medium. Here we provide evidence for the alternative possibility, that costimulation through integrin $\alpha_V\beta_3$ is essential for several readouts of responsiveness. In the absence of costimulation a partial signal is transduced, leading to uncompromised induction of Fas but not to secretion of IL-2 or apoptosis. Coligation by vitronectin induces IL-2 secretion and apoptosis, and osteopontin activates only IL-2 generation, while in combination osteopontin and vitronectin may act as competitors. These observations suggest the association of modular component pathways with the TCR (16, 26–29), some of which are blocked at checkpoints and depend on facilitation by suitable costimuli.

Various receptors are associated with multiple signal transduction modules which, in T-cells, may selectively predispose to proliferation or programmed cell death. This has been demonstrated for the IL-2 receptor that can mediate proliferation through Akt and Bcl-2 or can predispose to Fas-induced apoptosis after activation of Stat5 with consecutive FasL expression (30). Similarly, signal transduction through the TNF receptor triggers apoptosis in the presence of the inducibly expressed serine–threonine kinase RIP. In the absence of RIP, TNF induces T-cell activation (31). Engagement of the T-cell antigen receptor is also associated with two outcome phenotypes, but here costimulation determines which one predominates.

Ligation of integrin $\alpha_V\beta_3$ may have several biological consequences. Like other integrins, it can contribute to cell motility and adhesion by mediating haptotaxis followed by attachment and spreading (32–34). The interaction between extracellular matrix proteins and integrin receptors may also prevent a form of apoptosis frequently referred to as anoikis (35, 36). Integrins may cooperate with growth factors to enhance mitogenic signaling (37–39). In T-cell activation, ligation of the TCR corresponds to the engagement of a growth factor receptor. Our data suggest the existence of at

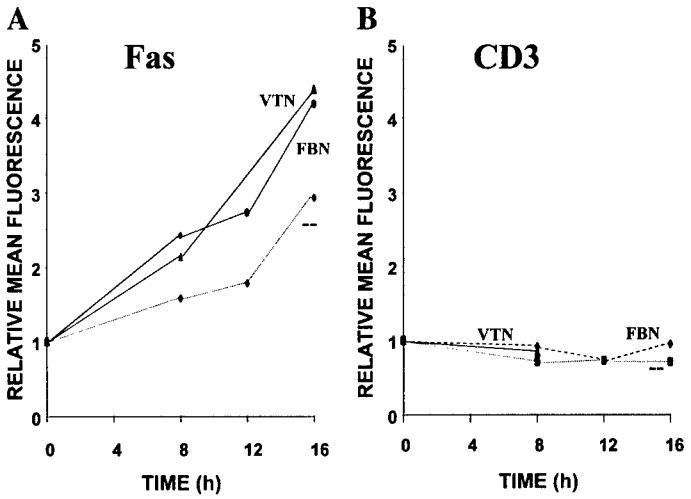

FIG. 5. Time course of surface marker expression in the presence or absence of costimuli. AF3.G7 cells were incubated in serum-free medium with plated anti-CD3 in the presence or absence of vitronectin or fibronectin for the indicated times. Surface marker expression of Fas (A) and CD3 (B) was analyzed by flow cytometry after staining with phycoerythrin-labeled antibodies and the results are expressed as relative fluorescence units. At each time point, cells stained with phycoerythrin-labeled anti-CD8 antibody served as negative controls.

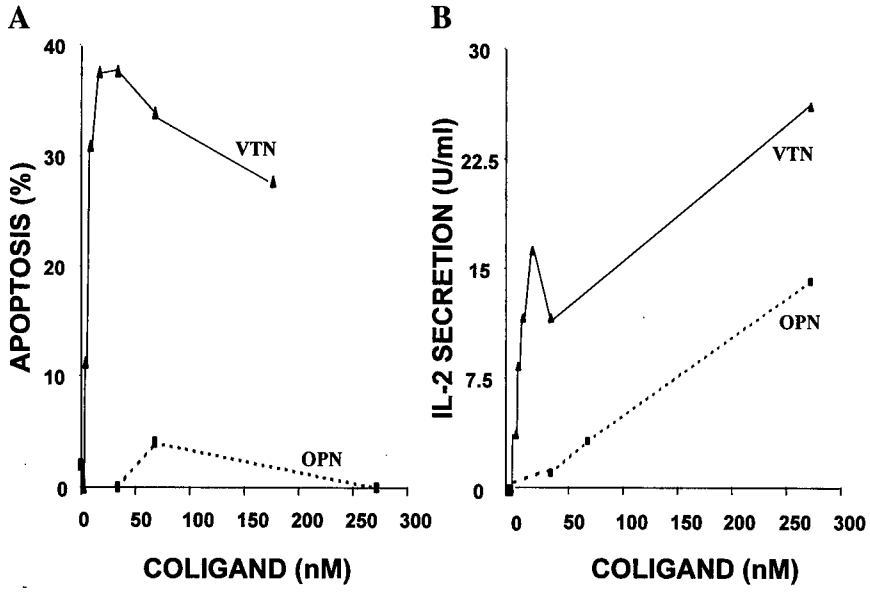

FIG. 6. Costimulation in primed peripheral $CD4^+$ cells. Lymph node derived $CD4^+$ cells were primed in complete medium with concanavalin A for 3 days and expanded in IL-2-containing medium for 4 consecutive days. The cells were stimulated in serum-free medium with plated anti-CD3 in the presence or absence of soluble extracellular matrix proteins as indicated. Apoptosis (A) and IL-2 secretion (B) depend on costimulation. In conformity to the data obtained with T-cell lines, costimulation of peripheral $CD4^+$ T-cells with vitronectin leads to apoptosis and IL-2 secretion while costimulation with osteopontin mediates IL-2 secretion but not apoptosis.

least three distinct pathways to T-cell activation. Induction of Fas is directly coupled to the T-cell antigen receptor (Fig. 5), is calcium independent, and is likely mediated by PKC through TDAG51 (40). IL-2 secretion depends on coligation of TCR and integrin receptors (Fig. 3). This activating TCR-dependent pathway may recruit PI 3-kinase through TCR-SHP2-Grb2 and involve activation of the transcription factors AP-1 and NF-AT which are responsible for IL-2 induction. This pathway can be enhanced by recruitment of PI 3-kinase to integrins through FAK (41–45) and may be engaged by osteopontin (46) or entactin. In contrast, signal transduction leading to T-cell apoptosis (Fig. 3) may advance via Lck, a p32 G-protein, phospholipase A, and downstream generation of hydroxyl radical (16, 47–49). Phospholipase A pathways have previously been associated with integrin signaling (49, 50) and may be engaged after coligation of integrin $\alpha_V\beta_3$ by vitronectin, fibronectin, or fibrinogen. Synergistically with the above, calcium-mediated or ERK/JNK-mediated signal transduction may lead to cell proliferation dependent on contributions from costimuli.

T-cells bearing the γ/δ receptor are frequently identified at sites of inflammation and may play an immunoregulatory rather than an effector function. Interestingly, the accessory contribution of integrin $\alpha_V\beta_3$ may play an even more dominant role in some subsets of γ/δ T-cells than in α/β T-cells because there it has become independent of ligation of the TCR. The associated signal transduction utilizes a pathway associated with the TCR ζ-chain (51, 52). If differential induction of activation and apoptosis also occurs in the γ/δ T-cell compartment this may have implications for autoimmune disease where the participation of these T-cells is often prominent.

Even though antigen specificity determines the target of an immune response, additional endogenous signals are required to define the type of immunity mounted. Most obviously, the presentation of an antigen by MHC I, MHC II, or noncanonical antigen-presenting molecules (such as CD1 or Qa-1) engages either $CD4^+$ or $CD8^+$ T-cells or NK cells. In addition, the prevalent cytokine profile, induced primarily by secretion of IL-10 or IL-12 from macrophages, can direct the immune system to antibody-mediated type II reactions or to cellular type I reactions. Accessory signals may also instruct individual T-cells to display specific response phenotypes. Coligation of CD28 on target T-cells by either B7 or CTLA4 concomitantly with antigen presentation determines whether the T-cells become responsive or anergized. Here we show that secreted extracellular matrix proteins may act as important costimulatory cytokines, which determine whether activated T-cells prevail or succumb to apoptosis. These molecules are ubiquitous in the extracellular matrix. There, they may serve largely structural roles, which do not seem to be essential because knockout mice for vitronectin or osteopontin do not display any developmental defects or connective tissue diseases (53, 54). When released from the matrix or secreted by immune cells, however, these molecules adopt cytokine characteristics and, expectedly, the costimulatory properties hinge on soluble presentation. The lack of measurable T-cell activation in the absence

of any of these cytokines reflects their important immunoregulatory function.

The expression of integrin $\alpha_V\beta_3$ on naive T-cells is low, but is induced on memory cells; therefore, the mechanisms described in this study may predominantly affect the repertoire of memory T-cells and imply a mechanism of control for immune responses by the memory compartment. Consistently, spleen and lymph node cells of osteopontin knockout mice display moderately elevated levels of CD62L and reduced levels of the memory markers CD45RB and CD44 (Weber, unpublished observations). Clinical implications derived from this include synovial T-cell survival in rheumatoid arthritis which may be mediated by anti-apoptotic effects of stromal cells exerted through T-cell integrins (8). HIV tat has been shown to ligate integrin $\alpha_V\beta_3$ on human T-cells (55, 56). This engagement activates the cells and facilitates infection (57). It may also lead to apoptosis and thus contribute to accelerated loss of $CD4^+$ cells, especially in the memory compartment where expression levels of integrin $\alpha_V\beta_3$ are high (58).

Note added in proof. Recently, Zheng *et al.* (*J. Biol. Chem.* **275,** 24565–24574) have shown that osteopontin and vitronectin can differentially activate migration and activation of the phosphatidylinositol 3-kinase/AKT pathway after ligation of integrin $\alpha_V\beta_3$ in prostate cancer cells.

ACKNOWLEDGMENTS

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REFERENCES

1. Manickasingham, S. P., Anderton, S. M., Burkhart, C., and Wraith, D. C., Qualitative and quantitative effects of CD28/B7-mediated costimulation on naive T cells *in vitro*. *J. Immunol.* **161,** 3827–3835, 1989.
2. Tao, X., Constant, S., Jorritsma, P., and Bottomly, K., Strength of TCR signal determines the costimulatory requirements for Th1 and Th2 CD4+ T cell differentiation. *J. Immunol.* **159,** 5956–5963, 1997.
3. Muraille, E., Devos, S., Thielemans, K., Urbain, J., Moser, M., and Leo, O., Costimulation regulates the kinetics of interleukin-2 response to bacterial superantigens. *Immunology* **89,** 245–249, 1996.
4. Anderson, D. E., Ausubel, L. J., Krieger, J., Hollsberg, P., Freeman, G. J., and Hafler, D. A., Weak peptide agonists reveal functional differences in B7-1 and B7-2 costimulation of human T cell clones. *J. Immunol.* **159,** 1669–1675, 1997.
5. Saukkonen, J. J., Tantri, A., and Berman, J., Costimulation of $CD28^-$ T cells through CD3 and beta1-integrins induces a limited Th1 cytokine response. *Scand. J. Immunol.* **50,** 145–149, 1999.
6. Bachmann, M. F., McKall-Faienza, K., Schmits, R., Bouchard, D., Beach, J., Speiser, D. E., Mak, T. W., and Ohashi, P. S., Distinct roles for LFA-1 and CD28 during activation of naïve T-cells: Adhesion versus costimulation. *Immunity* **7,** 547–557, 1997.
7. Vallejo, A. N., Mugge, L. O., Klimiuk, P. A., Weyand, C. M., and Goronzy, J. J., Central role of thrombospondin-1 in the activation and clonal expansion of inflammatory T cells. *J. Immunol.* **164,** 2947–2954, 2000.
8. Ng, T. T., Kanner, S. B., Humphries, M. J., Wickremasinghe, R. G., Nye, K. E., Anderson, J., Khoo, S. H., and Morrow, W. J., The integrin-triggered rescue of T lymphocyte apoptosis is blocked in HIV-1-infected individuals. *J. Immunol.* **158,** 2984–2999, 1997.
9. Salmon, M., Scheel-Toellner, D., Huissoon, A. P., Pilling, D., Shamsadeen, N., Hyde, H., D'Angeac, A. D., Bacon, P. A., Emery, P., and Akbar, A. N., Inhibition of T cell apoptosis in the rheumatoid synovium. *J. Clin. Invest.* **99,** 439–446, 1997.
10. Nicolls, M. R., Coulombe, M., Yang, H., Bolwerk, A., and Gill, R., Anti-LFA-1 therapy induces long-term islet allograft acceptance in the absence of IFN-gamma or IL-4. *J. Immunol.* **164,** 3627–3634, 2000.
11. Bohmig, G. A., Kovarik, J., Holter, W., Pohanka, E., and Zlabinger, G. J., Specific down-regulation of proliferative T cell alloresponsiveness by interference with CD2/LFA-3 and LFA-1/ICAM-1 *in vitro*. *J. Immunol.* **152,** 3720–3728, 1994.
12. Matsuyama, T., Yamada, A., Kay, J., Yamada, K. M., Akiyama, S. K., Schlossman, S. F., and Morimoto, C., Activation of CD4 cells by fibronectin and anti-CD3 antibody. A synergistic effect mediated by the VLA-5 fibronectin receptor complex. *J. Exp. Med.* **170,** 1133–1148, 1989.
13. Spinella, D. G., Hansen, T. H., Walsh, W. D., Behlke, M. A., Tillinghast, J. P., Chou, H. S., Whiteley, P. J., Kapp, J. A., Pierce, C. W., Shevach, E. M., *et al.* Receptor diversity of insulin-specific T cell lines from C57BL (H-2b) mice. *J. Immunol.* **138,** 3991–3995, 1987.
14. Gerstenfeld, L. C., Uporova, T., Schmidt, J., Strauss, P. G., Shih, S. D., Huang, L. F., Gundberg, C., Mizuno, S., and Glowacki, J., Osteogenic potential of murine osteosarcoma cells: Comparison of bone-specific gene expression in *in vitro* and *in vivo* conditions. *Lab. Invest.* **74,** 895–906, 1996.
15. McGahon, A. J., Seamus, J., Martin, Reid, P., Bissonette, Artin Mahoubi, Yu Fang Shi, Rana J. Magil, Walter K. Nishioka, Douglas R. Green. 1995. The end of the (cell) line: Methods for the study of apoptosis *in vitro*. *In* "Methods in Cell Biology, Vol. 46, Cell Death," (L. M. Schwartz and B. A. Osborne, Eds.). Academic Press, San Diego.
16. Weber, G. F., Abromson-Leeman, S., and Cantor, H., A signalling pathway coupled to T cell receptor ligation by MMTV superantigen leading to transient activation and programmed cell death. *Immunity* **2,** 363–372, 1995.
17. Sommer, F., Huber, M., Rollinghoff, M., and Lohoff, M., CD44 plays a co-stimulatory role in murine T-cell activation: Ligation of CD44 selectively co-stimulates IL-2 production, but not proliferation in TCR-stimulated murine Th1 cells. *Int. Immunol.* **7,** 1779–1786, 1995.
18. Huet, S., Groux, H., Caillou, B., Valentin, H., Prieur, A-M., and Bernard, A., CD44 contributes to T cell activation. *J. Immunol.* **143,** 798–801, 1989.
19. Shimizu, Y., van Seventer, G. A., Siraganian, R., Wahl, L., and Shaw, A., Dual role of the CD44 molecule in T cell adhesion and activation. *J. Immunol.* **143,** 2457–2463, 1989.
20. Ayroldi, E., Cannarile, L., Migliorati, G., Bartoli, A., Nicoletti, I., and Riccardi, C., CD44 (Pgp-1) inhibits CD3 and dexamethasone-induced apoptosis. *Blood* **86,** 2672–2678, 1995.

21. Ayroldi, E., Cannarile, L., and Riccardi, C., Modulation of superantigen-induced T-cell deletion by antibody Anti-Pgp-1 (CD44). *Immunology* **87,** 191–197, 1996.
22. Weber, G. F., Ashkar, S., Glimcher, M. J., and Cantor, H., Receptor–ligand interaction between CD44 and osteopontin (Eta-1). *Science* **271,** 509–512, 1996.
23. Weber, G. F., Adler, B., and Ashkar, S. Osteopontin in oxidative stress responses. *In* "Inflammatory Cells and Mediators in CNS Diseases" (R. R. Ruffolo, Jr., G. Z. Feuerstein, A. J. Hunter, G. Poste, and B. W. Metcalf, Eds.), pp. 97–112. Harwood Academic, Amsterdam, 1999.
24. Ashkar, S., Weber, G. F., Panoutsakopoulou, V., Sanchirico, M. E., Janssen, M., Zawaideh, S., Rittling, S., Denhardt, D. T., Glimcher, M. J., and Cantor, H., The cytokine Eta-1/Opn is an essential molecular bridge to type 1 (cell-mediated) immunity. *Science* **287,** 860–864, 2000.
25. Gerber, D. J., Pereira, P., Huang, S. Y., Pelletier, C., and Tonegawa, S., Expression of αv and β3 integrin chains on murine lymphocytes. *Proc. Natl. Acad. Sci. USA* **93,** 14698–14703, 1996.
26. Letourneur, F., and Klausner, R. D., Activation of T cells by a tyrosine kinase activation domain in the cytoplasmic tail of CD3 epsilon. *Science* **255,** 79–82, 1992.
27. Weber, G. F., and Cantor, H., Phosphatidylinositol synthesis is a proximal event in intracellular signaling coupled to T cell receptor ligation. *J. Immunol.* **152,** 4433–4443, 1994.
28. Weber, G. F., Mirza, N. M., Yunis, E. J., Dubey, D., and Cantor, H., Localization and treatment of an oxidation-sensitive defect within the TCR-coupled signalling pathway that is associated with normal and premature immunologic aging. *Growth Dev. Aging* **61,** 191–207, 1997.
29. Adler, B., Weber, G. F., and Cantor, H., Activation of T cells by superantigen: Cytokine production but not apoptosis depends on MEK-1 activity. *Eur. J. Immunol.* **28,** 3749–3754, 1998.
30. Van Parijs, L., Refaeli, Y., Lord, J. D., Nelson, B. H., Abbas, A. K., and Baltimore, D., Uncoupling IL-2 signals that regulate T cell proliferation, survival, and Fas-mediated activation-induced cell death. *Immunity* **11,** 281–288, 1999.
31. Pimentel-Muinos, F. X., and Seed, B., Regulated commitment of TNF receptor signaling: A molecular switch for death or activation. *Immunity* **11,** 783–793, 1999.
32. Nasu, K., Ishida, T., Setoguchi, M., Higuchi, Y., Akizuki, S., and Yamamoto, S., Expression of wild type and mutated rabbit osteopontin in *Escherichia coli,* and their effects on adhesion and migration of P388D1 cells. *Biochem. J.* **307,** 257–265, 1995.
33. Liaw, L., Almeida, M., Hart, C. E., Schwartz, S. M., and Giachelli, C. M., Osteopontin promotes vascular cell adhesion and spreading and is chemotactic for smooth muscle cells in vitro. *Circ. Res.* **74,** 214–224, 1994.
34. Senger, D. R., and Perruzzi, C. A., Cell migration promoted by a potent GRGDS-containing thrombin cleavage fragment of osteopontin. *Biochim. Biophys. Acta* **1314,** 13–24, 1996.
35. Frisch, S. M., and Ruoslahti, E., Integrins and anoikis. *Curr. Opin. Cell Biol.* **9,** 701–706, 1997.
36. Petitclerc, E., Stromblad, S., von Schalscha, T. L., Mitjans, F., Piulats, J., Montgomery, A. M., Cheresh, D. A., and Brooks, P. C., Integrin alpha(v)beta3 promotes M21 melanoma growth in human skin by regulating tumor cell survival. *Cancer Res.* **59,** 2724–2730, 1999.
37. Vuori, K., and Ruoslahti, E., Association of insulin receptor substrate-1 with integrins. *Science* **266,** 1576–1578, 1994.
38. Miyamoto, S., Teramoto, H., Gutkind, J. S., and Yamada, K. M., Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: Roles of integrin aggregation and occupancy of receptors. *J. Cell Biol.* **135,** 1633–1642, 1996.
39. Lin, T. H., Chen, Q., Howe, A., and Juliano, R. L., Cell anchorage permits efficient signal transduction between ras and its downstream kinases. *J. Biol. Chem.* **272,** 8849–8852, 1997.
40. Wang, R., Zhang, L., Yin, D., Mufson, R. A., and Shi, Y., Protein kinase C regulates Fas (CD95/APO-1) expression. *J. Immunol.* **161,** 2201–2207, 1998.
41. Eder, A. M., Dominguez, L., Franke, T. F., and Ashwell, J. D., Phosphoinositide 3-kinase regulation of T cell receptor-mediated interleukin-2 gene expression in normal T cells. *J. Biol. Chem.* **273,** 28025–28031, 1998.
42. Frearson, J. A., and Alexander, D. R., The phosphotyrosine phosphatase SHP-2 participates in a multimeric signaling complex and regulates T cell receptor (TCR) coupling to the Ras/mitogen-activated protein kinase (MAPK) pathway in Jurkat T cells. *J. Exp. Med.* **187,** 1417–1426, 1998.
43. Troussard, A. A., Tan, C., Yoganathan, T. N., and Dedhar, S., Cell-extracellular matrix interactions stimulate the AP-1 transcription factor in an integrin-linked kinase- and glycogen synthase kinase 3-dependent manner. *Mol. Cell. Biol.* **19,** 7420–7427, 1999.
44. Giancotti, F. G., and Rouslahti, E., Integrin signaling. *Science* **285,** 1028–1032, 1999.
45. Zell, T., Kivens, W. J., Kellermann, S. A., and Shimizu, Y., Regulation of integrin function by T cell activation: Points of convergence and divergence. *Immunol. Res.* **20,** 127–145, 1999.
46. O'Regan, A. W., Chupp, G. L., Lowry, J. A., Goetschkes, M., Mulligan, N., and Berman, J. S., Osteopontin is associated with T cells in sarcoid granulomas and has T cell adhesive and cytokine-like properties *in vitro. J. Immunol.* **162,** 1024–1031, 1999.
47. Mire-Sluis, A. R., Hoffbrand, A. V., and Wickremasinghe, R. G., Evidence that guanine–nucleotide binding regulatory proteins couple cell-surface receptors to the breakdown of inositol-containing lipids during T-lymphocyte mitogenesis. *Biochem. Biophys. Res. Commun.* **148,** 1223–1231, 1987.
48. Telfer, J. C., and Rudd, C. E., A 32-kD GTP-binding protein associated with the CD4–p56lck and CD8–p56lck T cell receptor complexes. *Science* **254,** 439–441, 1991.
49. Auer, K. L., and Jacobson, B. S., Beta 1 integrins signal lipid second messengers required during cell adhesion. *Mol. Biol. Cell.* **6,** 1305–1313, 1995.
50. Clark, E. A., and Hynes, R. O., Ras activation is necessary for integrin-mediated activation of extracellular signal-regulated kinase 2 and cytosolic phospholipase A2 but not for cytoskeletal organization. *J. Biol. Chem.* **271,** 14814–14818, 1996.
51. Roberts, K., Yokoyama, W. M., Kehn, P. J., and Shevach, E. M., The vitronectin receptor serves as an accessory molecule for the activation of a subset of γ/δ T cells. *J. Exp. Med.* **173,** 231–240, 1991.
52. Sturmhoefel, K., Brando, C., Martinon, F., Shevach, E. M., and Coligan, J. E., Antigen-independent, integrin-mediated T cell activation. *J. Immunol.* **154,** 2104–2111, 1995.
53. Liaw, L., Birk, D. E., Ballas, C. B., Whitsitt, J. S., Davidson, J. M., and Hogan, B. L., Altered wound healing in mice lacking a functional osteopontin gene (spp1). *J. Clin. Invest.* **101,** 1468–1478, 1998.
54. Zheng, X., Saunders, T. L., Camper, S. A., Samuelson, L. C., and Ginsburg, D., Vitronectin is not essential for normal mammalian development and fertility. *Proc. Natl. Acad. Sci. USA* **92,** 12426–12430, 1995.

55. Brake, D. A., Debouck, C., and Biesecker, G., Identification of an Arg-Gly-Asp (RGD) cell adhesion site in human immunodeficiency virus type 1 transactivation protein, tat. *J. Cell. Biol.* **111,** 1275–1281, 1990.

56. Zauli, G., Gibellini, D., Celeghini, C., Mischiati, C., Bassini, A., La Placa, M., and Capitani, S., Pleiotropic effects of immobilized versus soluble recombinant HIV-1 Tat protein on CD3-mediated activation, induction of apoptosis, and HIV-1 long terminal repeat transactivation in purified CD4+ T lymphocytes. *J. Immunol.* **157,** 2216–2224, 1996.

57. Li, C. J., Ueda, Y., Shi, B., Borodyansky, L., Huang, L., Li, Y. Z., and Pardee, A. B., Tat protein induces self-perpetuating permissivity for productive HIV-1 infection. *Proc. Natl. Acad. Sci. USA* **94,** 8116–8120, 1997.

58. Schnittman, S. M., Lane, H. C., Greenhouse, J., Justement, J. S., Baseler, M., and Fauci, A. S., Preferential infection of CD4+ memory T cells by human immunodeficiency virus type 1: Evidence for a role in the selective T-cell functional defects observed in infected individuals. *Proc. Natl. Acad. Sci. USA* **87,** 6058–6062, 1990.

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Review

The metastasis gene osteopontin: a candidate target for cancer therapy

Georg F. Weber *

Department of Radiation Oncology, New England Medical Center and Tufts University Medical School, 750 Washington Street, NEMC #824, Boston, MA 02111, USA

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Abstract

Malignant tumors are characterized by dysregulated growth control, overcoming of replicative senescence, and metastasis formation. Current therapeutic regimens mostly exert their effects through inhibition of cell cycle progression, leaving two major components of transformation untouched. The cytokine osteopontin is essential for the dissemination of various cancers. Past research has implied several modes in which osteopontin and its main receptors on tumor cells can be suppressed. Osteopontin expression is inhibitable on the levels of gene transcription and the RNA message, and the osteopontin protein can be blocked with antibodies or synthetic peptides. The osteopontin receptor CD44 has been targeted by diverse therapeutic strategies, including cytotoxic and immunotherapeutic approaches. The receptor integrin $\alpha_V\beta_3$ contributes not only to tumor cell dissemination, but also to angiogenesis and osteolysis in bone metastases. Small molecule inhibitors of this receptor are under study as drug candidates. Because receptors and cytokine ligands that mediate metastasis formation are sparsely expressed in the adult healthy organism and are more readily reached by pharmaceuticals than intracellular drug targets they may represent a particularly suitable focus for therapeutic intervention.

Keywords: Osteopontin; Metastasis; Cancer therapy; CD44; Integrin; Gene expression

1. Metastasis formation as a target for cancer therapy

The mortality of cancer is largely caused by locally and distally invasive growth. Transformed cells without the ability to grow destructively would form only benign tumors, which in most cases are eminently treatable. On the molecular level, the metastatic phenotype is generated by the expression of homing receptors with associated signaling molecules, their ligands, and extracellular matrix-degrading proteases. They represent gene products that are not derived from organogenesis, but are physiologically used by macrophages and lymphocytes for homing and for the induction of neovascularization in stress responses [1]. The first targets in metastatic spread are typically draining lymph nodes. Homing to and expansion in the lymphoid system corroborate the notion that cancer metastasis is based on mechanisms normally employed by immunocytes [2]. The differentiation of immune cells proceeds in the context of their tissue of residence so that lymphocytes from Peyer's patches are distinct from cutaneous lymphocytes and Kupffer cells are distinct from alveolar macrophages. Accordingly, recognition of topology may be encoded in the surface molecules of immune cells. Unlike the classical oncogenes (fre-

* Fax: +1-617-636-1766.
E-mail address: gweber@lifespan.org (G.F. Weber).

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quently affected by point mutations, deletions, frame shifts, or translocations) the genes involved in cancer spread have not been observed to be mutated in malignancies, although they are subject to dysregulation on the levels of transcription or splicing. In the adult healthy organism, expression of these molecules is typically rare. This makes them good candidate targets for cancer therapy.

Malignant transformation depends on three basic molecular mechanisms, proliferation, overcoming of replicative senescence, and metastatic spread. All would be suitable objects for anticancer agents. In contrast, conventional chemo- and radiation therapy aims at cell proliferation but is mostly not suitable to inhibit the mechanisms of cancer dissemination. It consists predominantly of radiation induced DNA damage, redox-cycling antibiotics (inducers of DNA strand breaks), antimetabolites (false building blocks), alkylating agents (DNA cross-linkers), cell cycle inhibitors (including spindle poisons), and in selected cases hormones. This deficit in the pharmacological armamentarium is highlighted by comparison with combination chemotherapy regimens of other diseases, which incorporate drugs that interfere with multiple pathogenic processes. Modern combination chemotherapy for infectious diseases has various targets and the drugs used in combination typically aim at more than one of them. (Example AIDS: non-nucleoside reverse transcriptase inhibitors, nucleoside analogs, protease inhibitors; highly active anti-retroviral therapy, or HAART, combines two to three nucleoside analogs with one or two protease inhibitors or one reverse transcriptase inhibitor. Example tuberculosis: isoniazid interferes with NAD^+ synthesis, rifampicin and ethambutol inhibit RNA synthesis, streptomycin interferes with RNA translation to protein; combination therapy combines several antibiotics to avoid the development of resistance.) Expansion of the spectrum of targets in cancer therapy holds the promise of enhancing efficacy and reducing unwanted effects. The increasing comprehension of molecular interactions involved in the dissemination of cancer cells should allow for the identification of promising drug targets. Nevertheless, few drugs have been designed which are suitable to suppress metastasis formation. Taxol and vincristine may inhibit cell motility through their effects on microtubules. The various anti-angiogenic drugs that are currently undergoing clinical tests also target dissemination. Bisphosphonates are in clinical use against bone metastases of multiple primary tumors.

The cytokine osteopontin contributes substantially to metastasis formation by various cancers. The following is a discussion of potential strategies for targeting osteopontin-dependent mechanisms of tumor cell dissemination in anti-neoplastic therapy. While most of these strategies have been used only in experimental research settings, often as scientific tools rather than as therapeutic agents, they may still point the way to future clinical approaches. Some methods proposed in this discourse are hypothetical insofar as they have not yet been specifically targeted to osteopontin. They may represent future potential that warrants experimental evaluation. The molecular connections that govern osteopontin induction and osteopontin effects through its various receptors are described in some detail. This is based on the notion that mechanistic insight is a first step toward a rational design of therapeutic intervention.

2. The roles of osteopontin in stress responses and in cancer dissemination

Osteopontin is an acidic glycoprotein with a protein backbone of about 32.5 kDa. It is rich in aspartate, glutamate and serine and contains about 30 monosaccharides, including 10 sialic acids. Carbohydrate is present as one *N*-glycosyl and five to six *O*-glycosyl side chains. Phosphorylation occurs to a variable extent, possibly on up to 28 sites [3], distributed throughout the molecule [4–6]. Osteopontin is produced by osteoclasts, macrophages, T-cells, kidneys, and vascular smooth muscle cells. It contributes to macrophage homing and cellular immunity. It also mediates neovascularization and inhibits apoptosis. Finally, osteopontin maintains the homeostasis of free calcium. Some of these functions of osteopontin are described in more detail below.

1. Osteopontin supports host resistance by inducing immune cell migration and invasion to sites of inflammation. Its primary target cell is the macrophage [7,8]. Osteopontin acts to mediate chemotaxis through interaction with CD44 [9] or hapto-

taxis through binding to integrin receptors [10]. The same mechanisms are utilized by tumor cells in the process of metastasis formation [1].

2. Osteopontin is an essential and early acting cytokine in cellular immune responses, it induces macrophages to mediate interleukin (IL)-10 suppression through interaction with CD44 and IL-12 secretion through binding to integrin $\alpha_V\beta_3$ [11]. These components are essential for cellular immunity and scarless wound healing. The osteopontin gene is associated with host resistance to infections with parasites, bacterial and viral pathogens [12,13] and maps to the Ric locus that confers resistance to the obligate intracellular bacterium *Rickettsia tsutsugamushi*, which is the etiologic agent for scrub typhus [12]. Specifically, the cytokine contributes to host defense against mycobacterial infection, it is elevated in granulomas caused by mycobacterium tuberculosis [13], and high levels of expression are a good prognostic indicator [14]. Bacillus Calmette-Guérin grows more rapidly in macrophages derived from osteopontin-deficient mice than in those from wild-type mice, which indicates an intrinsic macrophage defect [15]. Another aspect of this osteopontin function is the activation of immune system cells during wound healing. Following skin incisions, osteopontin is upregulated within 6 h after wounding. In the absence of osteopontin gene products, a significantly decreased level of debridement occurs, with greater disorganization of matrix and an alteration of collagen fibrillogenesis leading to small diameter collagen fibrils [16].
3. Osteopontin selectively contributes to stress-dependent angiogenesis. This form of neovascularization occurs after injury or hypoxia, is largely independent of anatomical borders, and is induced by a set of genes that is overlapping with, but distinct from the set of genes, which induces blood vessel formation during development. Osteopontin is one of three genes differentially expressed during in vitro angiogenesis [17]. In vascular smooth muscle cells, osteopontin may be induced by angiotensin II or catecholamines, both of which stimulate vascular growth [18,19]. Osteopontin receptors also contribute to the process, because a splice variant of CD44 is involved in endothelial cell proliferation and migration [20,21], and coordinate expression of β_3-integrins and osteopontin by regenerating endothelial cells can stimulate migration [22]. This may be facilitated by vascular endothelial growth factor-induced thrombin cleavage of osteopontin leading to enhanced induction of integrin $\alpha_V\beta_3$-dependent endothelial cell migration [23]. The engagement of integrin $\alpha_V\beta_3$ on endothelial cells by osteopontin prevents apoptotic cell death through activation of nuclear factor κB (NF-κB) p65 and p50, dependent on Ras and Src activation, but independent of signal transduction by mitogen-activated protein (MAP) kinase kinase or phosphatidylinositol 3-kinase (PI 3-kinase) [24]. Antagonists of integrin $\alpha_V\beta_3$ promote tumor regression by inhibiting neovascularization [25,26].
4. Osteopontin prevents apoptosis after a wide range of stresses [27]. The protection from apoptosis aids homing immune cells in reaching their destination. By counteracting a form of programmed cell death, often referred to as anoikis, the anti-apoptotic properties of osteopontin may also contribute to anchorage-independent growth of tumor cells [28]. Activated macrophages secrete reactive oxygen intermediates after phagocytosis. This oxidative stress, beside killing germs, causes peroxidation of membrane lipids with consecutive apoptosis of the phagocyte itself. Osteopontin is induced by macrophages concomitantly with phagocytosis and reduces the level of ensuing programmed cell death through anti-oxidant effects in an autocrine fashion [27]. Osteopontin also regulates apoptosis in T-lymphocytes. In conjunction with ligation of the T-cell antigen receptor, it acts as a co-stimulus that prevents programmed cell death [32]. The cytokine ameliorates damage after ischemia or reperfusion [29–31]. Osteopontin reverses the phorbol ester induced apoptosis in breast cancer cells that overexpress protein kinase C (PKC) α [33]. The anti-apoptotic effect depends on phosphorylation of osteopontin and can be reversed by anti-integrin β_3 antibodies [27].

Extracellular matrix molecules that contribute to cell invasion and metastasis formation typically have a modular structure, consisting of multiple functional domains that can be activated by post-translational modifications. Osteopontin is divisible

into multiple domains (Fig. 1) and its function is extensively regulated at the posttranslational level. The molecule has a protease hypersensitive site that separates the integrin and CD44 binding domains. The thrombin cleavage motif in this region has a conserved sequence, RSK, present in most species [34]. While binding of the C-terminal osteopontin fragment to CD44v6 occurs through a protein–protein interaction [9,11] it may also bind CD44v3 via a heparin bridge. In contrast, activation of the RGD-containing N-terminal domain is a prerequisite for integrin ligation. Cleavage by thrombin and phosphorylation of the N-terminal fragment are required for efficient engagement of the integrin receptor $\alpha_V\beta_3$ [11,35]. Osteopontin serves as a substrate for liver transglutaminase as well as the plasma transglutaminase factor XIIIa [36,37]. The catalyzed reaction modifies osteopontin on glutamines 34 and 36 [38], leading to the formation of γ-glutamyl-ε-lysine and linking osteopontin to specific extracellular locations. This has been associated with extracellular matrix remodeling [39] and may influence its haptotactic properties that are mediated by integrins. During mineral formation, osteopontin may incorporate significant amounts of sulfate. This suggests that sulfation is a specific marker of the osteoblastic phenotype [40] and may be reduced in lytic bone metastases. Multiple forms of human osteopontin are generated by alternative splicing in the N-terminal portion.

Transformed cells are often characterized by abundant secretion of osteopontin [41,42]. They include breast cancer, prostate cancer, osteosarcoma, glioblastoma, and squamous cell carcinoma. This may reflect inducible expression that is secondary to transformation with oncogenes, such as v-*myc* or *ras* [43] and results in metastasis formation [44]. The importance of osteopontin in tumor dissemination is highlighted by gene transfer experiments. Transfection of cells with osteopontin increases their malignant phenotype [45], whereas transfection with antisense oligonucleotides yields populations with reduced malignant potential [46,47]. Furthermore, transformation of cells from osteopontin knockout mice by transfection with *ras* leads to impaired colony formation in soft agar and slower tumor growth

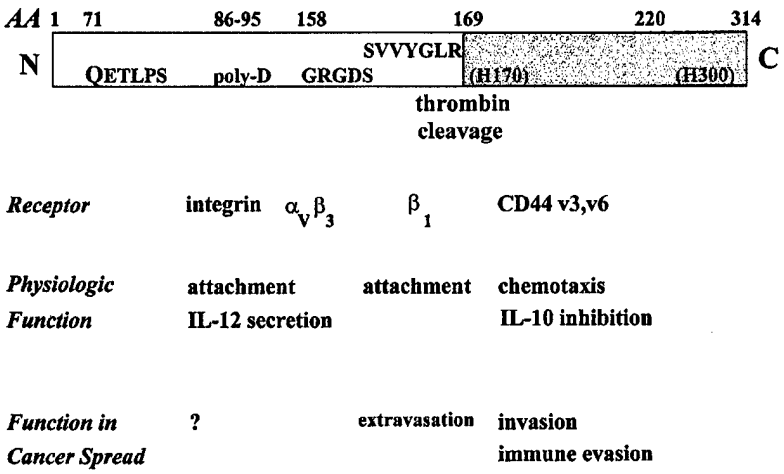

Fig. 1. Functional domains of the cytokine osteopontin. A poly-aspartate sequence in the N-terminal part of the molecule contributes to its acidity. In the middle of the molecule is a region with high sensitivity to multiple proteases, most notably to thrombin. Cleavage at this site splits the protein into two functional domains, the GRGDS-containing N-terminal fragment which ligates integrin receptors, predominantly $\alpha_V\beta_3$ and $\alpha_V\beta_5$, in a phosphorylation-dependent manner (the smallest functional domain identified reaches from amino acid 71 to 169), and the C-terminal fragment which may engage the homing receptor CD44 independently of posttranslational modifications (the smallest functional domain identified reaches from amino acid 169 to 220). While this polypeptide can ligate the variant exon 6 of CD44, the C-terminal fragment also contains two heparin binding sites (positions 170 and 300) which may form a bridge to the heparin binding site on variant exon 3 of CD44. The biological consequences of CD44v3 ligation remain to be elucidated. Between RGD motif and CD44 binding domain lies the recognition site for integrin β_1.

Table 1
Recognition elements and cognate transcription factors in the osteopontin promoter

Base	Recognition site	Transcription factors
First intron (enhancer)	palindromic recognition element	Oct-4
(adjacent to Oct-4 site)		Sox-2 repressor
Downstream of transcription initiation	inverted phorbol ester responsive site	AP-1
70 to 82		BPV-E2
−27 to −21	TATA box	
−39 to −34		Ets-1, in cooperation with PEBP2αA/CBFA1
−45 to −22	RE-1b	Oct-1, Oct-2
−53 to −49	v-Src response element	CBF-like factor
−86 to −55	RE-1a	Myc, Sp1, glucocorticosteroid receptor, E-box binding factor
−120 to −115	Pu box	Pu.1
−123 to +66		USF1
−180 to −229		Smad3
(adjacent to −180 to −229)		Smad4 competing with Hoxa-9 repressor
−270 to 259		PEA-3, IE1.2
−312 to −305		PEA-1
−413 to −405		PEA3/EBP20
−492 to −481		AP-2
−500 to −494		AP-4
−718 to −714		AP-1, FSE2.1
−725 to −712	Ras-activated enhancer sequence	MATF
−758 to −741	vitamin D response element	VDR/RXR
Not mapped	progesterone regulatory element	
Not mapped	SF-1 response element	ERRα, suppression by estrogen or PTH
Not mapped		Ets-2

Within the promoter for the osteopontin gene, multiple recognition sites for transcription factors have been mapped. The majority of them have been shown experimentally to be functionally relevant. The map was compiled from the references cited in the text.

the osteopontin promoter [74]), but not by estrogen receptor β [75]. These modulating effects may figure prominently in steroid hormone sensitive tumors, including breast and prostate carcinomas. Both malignancies metastasize predominantly to bone in a manner that depends on osteopontin expression. Systemic steroids are sometimes used as adjuvant analgesics in the treatment of cancer-related pain or in the symptomatic treatment of certain paraneoplastic syndromes. They may also be part of combination chemotherapy protocols of lymphomas. In the future, those regimens may need to be combined with measures that prevent osteopontin induction.

4.2. Signal transduction

Isoforms of PKC contribute to signal transduction in various cancer cells. Signaling through the EGF receptor involves PKC and induces osteopontin gene expression [65]. Osteopontin may also be induced as an early response gene in a PKC-mediated pathway in fibroblasts [76], osteoblasts [77], smooth muscle cells [78], and tumor cells [64,79]. Osteopontin is inducible by 12-*O*-tetradecanoylphorbol 13-acetate [80] and the promoter region of the osteopontin gene contains a phorbol ester responsive site (TGACTCA) in an inverted orientation downstream from the transcription initiation site [81]. A complex of the transcription factors and oncoproteins Jun and Fos binds to the phorbol ester responsive element leading to transactivation. The transcription factor AP-1, whose binding site is a highly conserved enhancer-like element present in many viral and cellular genes, is involved in the phorbol ester response. The tumor suppressive properties of several PKC inhibitors are under study and have the potential to suppress osteopontin expression in addition to growth suppression. Among them are Go6976, safingol, and calphostin C. 7-Hydroxystaurosporine (UCN-01) is an anti-tumor agent as well as a potent inhibitor of a

variety of protein kinases with a preference for PKC. The partially selective kinase inhibitor, PKC412 blocks substrate phosphorylation by several isoforms of PKC.

One proto-oncogene whose gain-of-function mutations induce upregulated transcription of osteopontin is *ras*. Transfection of NIH3T3 cells with *ras* leads to the induction of osteopontin and causes a malignant phenotype, whereas transfection of *ras* into LTA cells does not induce osteopontin expression and does not mediate malignancy [82]. Transfection of cloned rat embryonic fibroblast cells with H-*ras* causes transformation and expression of osteopontin and metalloproteinases. Co-expression of the tumor suppressor gene Krev-1 reverses the cellular phenotype and delays in vivo metastasis formation in a manner that is correlated with reduced osteopontin expression [83]. The augmented osteopontin gene expression in *ras*-transformed metastatic cells is mediated in part by the Ets-related transcription factor, MATF, which can bind to the Ras-activated enhancer sequence of the osteopontin promoter [45,84]. Therapeutic agents that interfere with Ras signal transduction, such as hypothemycin [85], may have a dual benefit against certain tumors by inhibiting cell cycle progression and osteopontin expression. Similarly, ribozyme mediated inhibition of *ras* expression [86] can block this pathway of osteopontin induction.

The osteopontin promoter contains recognition sites for multiple members of the Ets superfamily of proto-oncogenic transcription factors, including AP-3 and an Ets-1 site (bases −39 to −34) [45]. Proto-oncogenes of the Ets family have been associated with various carcinomas and their therapeutic inhibition could suppress osteopontin gene expression. The transcription factor PEBP2αA/CBFA1 cooperates with Ets1 to regulate expression of the osteopontin gene in skeletal tissue and a dominant negative PEBP2αA/CBFA1 diminishes the levels of osteopontin mRNA [87]. These transcription factors, therefore, may be suitable targets in the therapy of osteosarcoma.

Osteopontin expression is induced by activation of various cellular oncogenes. Expression of v-*src* in fibrosarcoma cells significantly stimulates osteopontin promoter activity through a v-Src response element, that is an inverted CCAAT box located at −53 to −49 bases. The mediator of this signal could be a CBF-like factor [88]. Consistently, cells from *src*$^{-/-}$ mice have diminished osteopontin expression [65]. Activity of AP-1/PEA-1 is stimulated by v-*src*, v-*mos*, v-*raf*, and c-*fos*. Like the AP-1 and PEA-1 sites (bases −718 to −714 and −312 to −305), the BPV-E2 (bases +70 to +82) binding site may also contribute to increased expression in cells with activated oncogenes. Therapeutic inhibition of these pathways may not only suppress growth but also dissemination.

Cells of the preimplantation embryo secrete osteopontin. At this stage, the POU transcription factor Oct-4 is expressed and binds to the first intron of osteopontin, which functions as an enhancer in immature cell lines. The palindromic Oct factor recognition element in the first intron is composed of an inverted pair of homeodomain binding sites separated by 5 bp (ATTTG +5 CAAAT). POU proteins are required for strong transcriptional activation of the osteopontin element, whereas the canonical octamer overlapping with the downstream half of the palindromic Oct factor recognition element is not essential. The transcription factor Sox-2 is co-expressed with Oct-4 in the early embryo. It represses Oct-4 mediated activation of osteopontin by way of a canonical Sox element that is located close to the palindromic Oct factor recognition element. Repression depends on a carboxy-terminal region of Sox-2 [89]. In embryonic carcinomas, suppression of Oct-4, conceivably by Sox-2-derived peptides, may represent a desirable therapeutic goal. A proximal promoter element (bases −94 to −24), designated RE-1, is essential for maintaining high levels of osteopontin expression in astrocytomas. It consists of two cis-acting elements, RE-1a (bases −86 to −55, containing binding sites for Myc, Sp1, the glucocorticosteroid receptor, and E-box binding factors) and RE-1b (bases −45 to −22, containing binding sites for Oct-1 and Oct-2), which act in synergism [90].

5. Osteopontin as a target for cancer therapy

The biological functions of metastasis associated gene products are extensively regulated at the post-transcriptional and posttranslational levels. Therefore, osteopontin from various cellular sources may

have diverse structural characteristics [91] that are reflected in distinct physiologic roles. The main source of osteopontin is $CD4^+$ T-cells. Macrophages may also produce osteopontin after stimulation with lipopolysaccharide but with distinguishable biological consequences. The macrophage-generated osteopontin is structurally different from the T-cell-derived molecule, possibly due to loss of part of its sequence by alternative splicing (S. Ashkar and G.F. Weber, unpublished observations). Studies in osteopontin$^{-/-}$ gene-targeted mice [92] have suggested the existence of structural and functional differences between tumor-derived osteopontin and the osteopontin forms that are relevant for host defenses. Evidence suggests that tumor-derived osteopontin is unique (i.e. structurally different from osteopontin derived from untransformed cells) and lacks important domains. As a case in point, an osteosarcoma secreted a smaller form of osteopontin than the predominant product secreted by non-transformed bone cells [93]. Malignant cells often secrete hypophosphorylated osteopontin variants [94] or a variant that has a deletion in its N-terminal portion [95] and this molecule may contribute to metastatic spread [96] by inducing cell migration. Concomitantly, hypophosphorylated or alternatively spliced osteopontin could ligate CD44 on macrophages leading to chemotaxis [9] and suppression of IL-10, but would not engage its integrin receptors so that IL-12 would not be efficiently secreted [11]. Thus, expression of structurally altered osteopontin by cancer cells may, among other functions, represent a mechanism of immune evasion.

5.1. Suppression of the osteopontin message

There are currently two principal strategies to suppress gene expression on the level of the RNA message, namely ribozyme cleavage (1) and hybridization with antisense oligonucleotides (2). In both cases, the mRNA is degraded before it can be translated into protein. However, both strategies are compromised by the difficulty of intracellular delivery of the nucleic acid drugs.

1. Small RNA endonucleases (ribozymes) reversibly cleave the phosphodiester bond of substrate RNA to generate 5′-hydroxyl and 2′,3′-cyclic phosphate termini, thus specifically inhibiting the expression of target genes. Transacting hammerhead ribozymes contain a catalytic domain and flanking regions, which allow hybridization to the target sequence. Short stretches of RNA (possibly as low as 19 nucleotides) may suffice to generate catalytic activity. Ribozymes are being tested for anti-cancer gene therapy with oncogenes, such as *ras, EGF receptor, bcl-2*, and *her-2*, as targets [97–102].
   The osteopontin message is amenable to targeting by ribosomal endonucleases. Three hammerhead ribozymes designed to cleave three different regions of osteopontin mRNA reduced osteopontin expression in a subset of transformed cells. For expression in vivo, the duplex DNA templates were cloned into a mammalian expression vector. Transfected cells had greater sensitivity to killing by macrophages and were less tumorigenic and metastatic [103].
2. Therapy with antisense oligonucleotides is intended to prevent the translation of proteins that are associated with a particular disease state. Antisense drugs have the potential for higher specificity and selectivity than small molecule drugs, because of binding to mRNA targets through multiple points of interaction at a single receptor site. By acting at this earlier stage, antisense may provide greater therapeutic benefit than traditional drugs, which do not act until the disease causing protein has been produced. In addition, the design of antisense drugs is less complex and more rapid than conventional drugs because it is restricted by the base sequence of the target RNA. Osteopontin antisense can be expressed by stable transfection with a mammalian expression vector containing the relevant cDNA in an inverted orientation. Expression of antisense osteopontin RNA in metastatic *ras*-transformed NIH3T3 mouse fibroblasts is associated with reduced malignancy. Primary tumor growth rates in nude mice as well as experimental metastases in a chick embryo metastasis assay and in nude mice were reduced or completely inhibited [46,104]. Antisense RNA caused a reduction in osteopontin secretion by malignant B77-Rat1 fibroblasts and reduced their ability to form both lung tumors in

nude mice after intravenous injection and colonies in soft agar. Antisense transfectants also showed increased spreading on vitronectin [47], suggesting that competition between osteopontin and vitronectin for the integrin receptor may contribute to modulating metastatic spread. Antisense inhibition of osteopontin expression may suffice to prevent full transformation. Several clonal lines of preneoplastic JB6 cells derived from BALB/c mouse epidermal cultures become irreversibly oncogenic and concomitantly synthesize osteopontin at elevated levels upon treatment with 12-*O*-tetradecanoyl phorbol-13-acetate. Stable transfection of these clones with inducible osteopontin antisense RNA prevented augmented osteopontin expression at both mRNA and protein levels following treatment, and cells from all four clones failed to form colonies in soft agar medium [105].

5.2. *Inhibition of the osteopontin protein*

Inhibition of protein action is most frequently attempted by antibodies or synthetic peptides. Even though difficulties of drug delivery compromise the use of peptide based therapeutics the problem is ameliorated when secreted molecules are targeted because cellular uptake of the peptide drug is not necessitated. Experiments with early inhibitors of metalloproteinases, based on the peptide structure of collagen, showed that they can significantly reduce the growth rate of primary and secondary tumors, and can block the process of metastasis formation [106]. Analogs of the hypothalamic hormones LH-RH and somatostatin can modulate their biological effects and are in use for cancer treatment [107]. Finally, controversial efforts have been made with naturally occurring peptides, referred to as antineoplastons, to control tumorous growth [108].

Various antibodies have been synthesized that recognize distinct epitopes of osteopontin [91,109]. Polyclonal antibodies to osteopontin inhibit the growth stimulatory effect of endogenous osteopontin in human prostate carcinoma cells [28]. A murine anti-human osteopontin antibody, which may recognize the RGD/thrombin cleavage region, inhibited the adhesion of MDA-MB-435 breast cancer cells [34]. Because prevention of adhesion of epithelial cells typically induces a form of apoptosis (often referred to as anoikis) it is possible that interference with cancer cell adhesion to osteopontin may suffice to induce programmed cell death, implying use for humanized versions of anti-osteopontin antibodies in cancer treatment.

The RGD sequence of osteopontin is essential for integrin engagement and is therefore conserved in all species. Cell adhesion is mediated by binding of the GRGDS motif in osteopontin to integrin receptors on tumor cells. Synthetic peptides containing the RGD motif are routinely used experimentally to block cellular interactions with osteopontin [110,111]. Phosphorylation of the serine in GRGDSL results in significant reduction of cell binding [112]. Similarly, insertion of α-methylserine next to the RGD sequence results in lower affinity for both stereoisomers and several small changes in chemical shifts and coupling constants relative to the parent serine peptide GRGDSL [113]. For therapeutic use, more specific sequences will have to be identified.

5.3. *Modulation of posttranslational modifications*

The extensive regulation of osteopontin function by posttranscriptional mechanisms and the inferred structural differences between tumor-derived and T-cell-secreted osteopontin may imply that their targeting by therapeutics would be desirable. Osteopontin would still be expressed and secreted, while some of its functions could be suppressed. For example, prevention of thrombin cleavage does not affect CD44 engagement, but prevents signaling through integrin $\alpha_V\beta_3$. This goal is compromised because thrombin, kinases, transglutaminase, and glycosyl transferases all have multiple substrates. Inhibition of their actions could alter the functions of other proteins.

Thrombin cleavage of osteopontin is a prerequisite for activation of its integrin binding domain. Signals transduced through the integrin receptor are essential for the osteopontin induced dissemination of various tumors. It is currently unknown whether there is a benefit in preventing osteopontin cleavage by thrombin in cancer. Diverse thrombin inhibitors are in clinical use [114–116] and could be tested in this regard.

Osteopontin is a substrate for casein kinase, fac-

tor-independent Golgi kinase, cAMP- and cGMP-dependent kinases [3,5,6,117–119]. Conversely, osteopontin is also a substrate for tartrate-resistant acid phosphatase. The phosphorylation status of osteopontin upon secretion is influenced by its mode of induction. 1α,25-Dihydroxy-vitamin D_3 and estrogens can both affect the extent of phosphorylation of secreted osteopontin. The functional consequences for tumor metastasis of phosphorylation of the multiple sites on the osteopontin protein have not yet been sufficiently studied to make them promising targets in cancer therapy.

Crosslinking of osteopontin by transglutaminase (*R*-glutaminyl-peptide:amine γ-glutamyltransferase, EC 2.3.2.13) may be a prerequisite for tumor cell haptotaxis. Osteocalcin, the most abundant non-collagenous protein of the bone matrix, has an inhibitory effect on tissue transglutaminase activity, as measured by cross-linking of osteopontin. The inhibitory activity resides within a peptide comprising the first 13 N-terminal amino acid residues of osteocalcin, which has affinity for transglutaminase substrates and exhibits homology to the substrate recognition sites of two transglutaminases. Therefore, the inhibitory effect is most likely due to competition with the enzyme for the transglutaminase binding region of the substrates, which prevents access of the enzyme to them. The interference of osteocalcin with osteopontin cross-linking gives osteocalcin a potential function as a protein inhibitor of tissue transglutaminase [120]. Chemical inhibitors of tissue transglutaminase are monodansylcadaverine and cystamine [121,122].

5.4. Protein engineering

The engineering of proteins has appeal because of the wide range of functions that can be induced, potentially going beyond the limits of what can be achieved by endogenous molecules. The promise of such approaches has to be balanced by their challenges, which include potential antigenicity of novel protein sequences and limited control over possible unwanted effects of structures that have no biological correlate. The fusion peptide designs described below are suited to immobilize transformed cells and may have applications in purging strategies.

Bone-specific extracellular matrix proteins contain amino acid sequences that mediate cell adhesion. Many of them also have acidic domains that interact with the mineral surface and may orient the signaling domains. Salivary statherin contains a 15-amino acid hydroxyapatite binding domain (N15) that is loosely helical in solution. N15-PGRGDS fusion peptides between RGD plus flanking residues from osteopontin and the C-terminus of salivary statherin bound tightly to hydroxyapatite and mediated the dose-dependent adhesion of melanoma cells when immobilized on the hydroxyapatite surface. The integrin $\alpha_V\beta_3$ was the primary receptor target for the fusion peptide. This fusion peptide framework may thus provide a design for immobilizing bioactive sequences on hydroxyapatite [123].

Streptavidin is widely used as an adapter molecule. Cell adhesive peptides, consisting of hexapeptide sequences derived from osteopontin and fibronectin that contain the RGD cell adhesion sequence, were engineered into the three-dimensional scaffolding of streptavidin. The mutations did not alter refolding or tetramer assembly and the slow biotin dissociation rate of wild-type streptavidin was retained. In cell binding assays, rat aortic endothelial cells and human melanoma cells adhered to surfaces coated with either of the two RGD streptavidin mutants dose-dependently and in a fashion that was inhibitable with soluble RGD peptide or with antibodies directed to integrin $\alpha_V\beta_3$, whereas wild-type streptavidin displayed no significant cell binding activity [124]. The observation that peptide recognition sequences can be engineered into accessible surface regions of streptavidin without disrupting its biotin binding properties implies potential utility in the trapping of cancer cells.

6. The receptor CD44 as target for cancer therapy

Osteopontin exerts its pro-metastatic effects through its respective receptors on tumors, in part through the CD44 splice variants 3 and 6. The interference with these receptor–ligand interactions on the level of inhibiting receptor expression, blocking receptor–ligand binding, or suppressing associated signal transduction has the potential to curtail the process of metastatic dissemination and thus to preempt the malignant phenotype.

6.1. Modulation of CD44 expression on tumor cells

Growth factors, such as TNF-α, may induce the expression of CD44 variants v3, v6, and v9 [125]. Similarly, oncogenes can regulate CD44 expression. The activated oncogene c-Ha-*ras* induces CD44 expression in cloned rat embryonic fibroblasts. NIH 3T3 cells transformed with either the Harvey or Kirsten *ras* oncogenes express much higher levels of cell surface CD44 protein than parental 3T3 cells, *ras* revertants generated from Kirsten *ras*-transformed cells, or c-*sis* transformants, confirming the significance of the *ras* oncogene in this upregulation [126]. Increased transcript levels for the standard form of CD44 are accompanied by the appearance of alternatively spliced RNAs and the synthesis of CD44 variants. The adenoviral gene product E1A counteracts the Ras-induced promoter function and, consequently, inhibits metastatic behavior without reversing transformation [127]. These connections emphasize the therapeutic potential for Ras inhibitors (see also Section 4.2).

6.2. Inhibition of CD44 ligation by osteopontin

In the absence of candidate small molecule drugs, three general strategies exist for therapeutic interference with receptor ligation. They include the use of antibodies (1), peptide antagonists (2), and soluble receptors (3).

1. Due to their high affinity binding, antibodies are often employed to block receptor–ligand interactions. Most prominently in cancer therapy, antibodies to HER-2 (herceptin) [128] have had substantial success against breast cancer. The malignant phenotype of a rat pancreatic adenocarcinoma is caused by a splice variant of CD44 containing variant exon 6 [50]. Metastasis formation in lymph nodes and lungs by this adenocarcinoma could be blocked by intravenous injection of an anti-variant CD44 antibody (mouse IgG_1). The antibody was only effective when given before lymph node colonization; however, one injection was sufficient to retard metastasis formation. The antibody is not cytopathic or cytotoxic by itself or via complement, it does not induce antibody-dependent cellular cytotoxicity, and it does not activate immunoglobulin-specific T-cells (this lack of T-cell activation has been attributed to blocking of CD44v on activated T-cells with possible immunosuppressive consequences). The antibody does not act by downregulating CD44 expression or by activation of an immune defense. The proposed mechanism of action is the inhibition of interaction between CD44v6 and its ligand [129], now known to be osteopontin.
2. Attempts have been made to utilize peptide antagonists of receptors in cancer therapy. Because they often model the binding domain of physiologic ligands, such peptide antagonists may have substantial specificity. However, the concentrations needed for effective blocking are often high, and parenteral application is mandatory to avoid destruction of the peptides in the stomach. Abarelix, a peptide antagonist of GnRH receptor, is being studied for the treatment of prostate cancer [130]. Native somatostatin has been viewed as a possible therapeutic for growth hormone- and thyrotropin-secreting tumors. Its short duration of action has been overcome with octreotide, a long-acting, synthetic octapeptide analog of the naturally occurring hormone [131]. We have localized the CD44 binding site of osteopontin to an RGD-independent 65 amino acid peptide [11] (S. Ashkar and G.F. Weber, unpublished results) (Fig. 1). Smaller peptides of this domain may possibly serve as blocking agents that inhibit this receptor/ligand interaction.
3. Soluble receptors can also be used to sequester growth factors from their specific membrane-bound receptors. Fas is expressed on the surface of a number of cell types. A Fas messenger RNA variant encoding a soluble molecule is generated by the deletion of the exon encoding the transmembrane domain. Soluble Fas blocks apoptosis induced by the antibody to Fas [132] or by Fas ligand. Likewise, the IL-2 receptor can be shed and elevated levels of serum soluble IL-2 receptor are a prominent feature of certain hematologic malignancies [133], including non-Hodgkin lymphoma [134].
   CD44 can be shed from the cell surface and act as a soluble molecule [135], following ectodomain cleavage, mediated through the pathway Ras → phosphoinositide 3-OH kinase → Cdc42/

Rac1 [136]. This is believed to reflect scavenging, by which the soluble receptor engages the physiologic ligands thus preventing them from binding to the receptor on the cell surface [137]. For the interaction with hyaluronate, the other major ligand for CD44, it has been demonstrated that transfection of mammary carcinoma cells with cDNA encoding soluble forms of CD44 inhibits the ability of endogenous cell surface CD44 to bind and internalize hyaluronate and to mediate invasion of hyaluronate-producing cell monolayers. Mice intravenously injected with soluble CD44-producing cells developed few or no tumors, whereas untransfected cells generated substantial pulmonary metastases. Both cell types initially adhered to pulmonary endothelium and penetrated the interstitial stroma, however, although parental cells divided and formed clusters within lung tissue, most of the transfectants underwent apoptosis [138]. While similar analyses have not been performed with regard to the interactions between CD44 and osteopontin, it is likely that secreted forms of CD44v3–6 will exert comparable inhibitory effects. This implies potential therapeutic use for engineered soluble CD44 molecules in cancer. Because shedding occurs through proteolytic cleavage of the extracellular portion of CD44 by chymotrypsin-like activity [139], it is conceivable that therapeutic application of a protease that sheds CD44 from the membrane would also inhibit dissemination by two mechanisms, removal of a surface receptor that is important for invasion and scavenging of the physiologic ligands by the released extracellular receptor domain.

6.3. Targeting of CD44 in cytotoxic therapy

Cytotoxicity is the common mechanism of most anti-cancer therapeutics that are in use today. This makes targeting to tumor cells and minimizing of the damage to bystander cells an urgent matter. The homing of a cytotoxic agent to a cancer can be facilitated by coupling it to a ligand that is bound or taken up selectively by the tumor cells. An epitope encoded by sequences of the CD44 variant exon 6 is recognized by the murine monoclonal IgG_1 VFF18 with high affinity. Application of radiolabeled VFF18 to nude mice bearing human epidermoid carcinoma xenografts led to fast and selective uptake of the radioimmunoconjugate into the tumor, making the antibody VFF18 a promising vehicle for cytotoxic therapy of squamous cell carcinomas [140]. While speculative at this point, it is conceivable that synthetic peptides of the CD44 binding domain of osteopontin or parts thereof could also serve as carrier molecules to target cytotoxic or cytostatic agents to the tumor. Such peptides, like osteopontin, are expected to be internalized following receptor ligation so that coupled anti-cancer drugs would accumulate within the tumor cells.

6.4. Targeting of CD44 in immunotherapy

The CD44 variants expressed on tumor cells have been the focus of immunotherapeutic strategies. Their role as tumor associated antigens is based on the infrequent expression of CD44 splice variants in the adult healthy organism. However, a potential problem of immunotherapy for metastasis associated gene products is that these molecules are themselves used by activated macrophages and lymphocytes (see Section 1). This form of immunotherapy could therefore attack activated immune system cells, thereby defeating its own purpose.

1. Bispecific antibodies are conjugates of Fab′ fragments, of which one recognizes a tumor antigen and another binds specifically to select immunologic effector cells. They were developed based on the hypothesis that strong persistent binding between immune system effector cells and tumor cells may enhance anti-tumor host defenses.
   A bispecific F(ab′)2 antibody conjugate was constructed against the complement receptor CR3 of macrophages and the CD44v6 antigen of rat pancreatic adenocarcinoma cells to redirect macrophage-mediated tumor cytotoxicity. The Fab′ fragments of monoclonal antibodies 1.1ASML and OX42, recognizing the CD44v6 and the CR3 antigens respectively, were chemically coupled at the hinge region. Although the monovalence resulted in lower avidities to both the antigens, it initiated the formation of stable cross-linkages between tumor cells and macrophages in culture leading to the formation of cell aggregates. The in vitro and in vivo tumor-targeting capacity

of the bispecific antibody conjugate was compared with that of the parental antitumor monoclonal antibody 1.1ASML, which mediates tumor killing by antibody-dependent cell cytotoxicity. Even though the bivalent monoclonal antibody 1.1ASML did not convey stable cross-linking of target and effector cells, its Fc-receptor-mediated killing of tumor cells was more effective when compared to the bispecific antibody conjugate. This supports the hypothesis that firm persistent binding between effector and target cells per se is not as important as the choice of the trigger molecule used for macrophage activation to direct their tumor cytotoxic potential effectively [141].

2. Cytotoxic T-lymphocytes are crucial in host defense due to their capacity to lyse selected target cells. Because recognition by the T-cell receptor depends on MHC-restricted antigen expression on the cell surface, the use of cytotoxic T-cells in the elimination of cancer is largely limited to cells that present neoantigens on their surface. This constraint can be circumvented by a procedure in which the ζ component of the T-cell receptor is genetically manipulated and equipped with an extracellular recognition domain. Introduction of a chimeric gene, consisting of the ζ chain of the T-cell receptor and a single chain antibody domain, into cytotoxic T-lymphocytes results in T-cells with a predetermined recognition specificity for particular tumor cells with the following characteristics. First, retrovirally mediated gene transduction used to introduce chimeric ζ chain constructs into in vitro activated T-lymphocytes leads to a high gene transduction efficiency into primary T-cells. Second, these primary T-cells assume a predetermined specificity for target cell recognition and lysis. Third, the MHC restriction of target cell recognition can be avoided and tumor cells recognized by the single chain antibody domain are lysed. Fourth, the production and provision of tumor cell-specific T-lymphocytes may not be sufficient to eradicate large tumors in vivo, because certain tumors secrete transforming growth factor β and thereby suppress lymphocyte activity [142].
The monoclonal antibody 1.1ASML, which recognizes a CD44v6 epitope, interferes with metastatic dissemination of a rat pancreatic carcinoma. The single chain antigen binding fragment of this antibody was fused to the ζ chain of the T-cell receptor complex and expressed in murine cytotoxic T-lymphocytes. The resulting cellular clones were not MHC-restricted in their CD44v6 recognition and exhibited in vitro lytic activity toward CD44v6 positive cells. Tumor cell xenografts grown in athymic nude mice were suppressed in their growth upon infusion of the genetically manipulated cytotoxic T-lymphocytes [143]. The CD44 variant exon 7 may laterally engage integrin β_1 in the cell membrane and act as an osteopontin receptor (see Section 7). An epitope encoded by sequences of the variant exons 7 and 8 is recognized by the monoclonal antibody VFF17. Its single chain antibody binding fragment was fused to the T-cell antigen receptor ζ chain and introduced into a cytotoxic T-cell line by retroviral gene transfer. Recombinant clones expressed the fusion protein on their cell surface and lysed CD44v7–8 expressing target cells specifically and MHC-independently. Cervical cancer cells, which express CD44v7–8, were efficiently lysed by these genetically engineered cytotoxic T-lymphocytes [144].

7. The receptor integrin $\alpha_V\beta_3$ as target for cancer therapy

The roles of integrin $\alpha_V\beta_3$ in cancer include invasion of tumor cells, osteoclast activation in lytic bone metastases, and neovascularization. The ligand osteopontin may contribute substantially to all functions and interference with their interaction may profoundly curtail the dissemination of various cancers.

7.1. Modulation of integrin $\alpha_V\beta_3$ expression on tumor cells

Exposure to steroids may suppress integrin $\alpha_V\beta_3$ expression. Regarding the use of steroids in cancer treatments, however, it is important to consider the multitude of effects on the host and the tumor (see Section 4.1). According to experiments with osteoblasts, short-term exposure of cells to dexamethasone may increase the expression of integrin $\alpha_V\beta_3$; however, long-term exposure to this glucocorticosteroid

downregulates integrin $\alpha_V\beta_3$ expression. In cell culture, levels of mRNA for the α_V chain are elevated during the first 1–3 days followed by inhibition. In contrast, mRNA levels for the β_3 chain are decreased at all time points. Furthermore, after an 8-day treatment, degradation of both RNA messages is accelerated [145].

The expression of integrin $\alpha_V\beta_3$ has been suppressed experimentally by transfection of a construct encoding an intracellular single chain antibody directed to the α_V chain. Saos-2 osteosarcoma cells were transfected with an expression plasmid coding for an intracellular single chain antibody to the α_V integrin subunit. They showed an approximately 70–100% decrease in the cell surface expression of integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$. Intracellular antibody expression had no effect on the integrin α_V mRNA levels and translation of the precursor α_V integrin subunit was not affected. However, the maturation of α_V integrins as glycoproteins was slow suggesting that the transport from endoplasmic reticulum to Golgi complex was partially prevented. Depletion of α_V integrins from Saos-2 cells led to a decreased ability to spread on fibronectin and vitronectin [146].

7.2. *Inhibition of receptor ligation by osteopontin*

As described above (Section 6.2), two principal strategies for inhibiting receptor ligation are based on the use of antibodies (1) and synthetic peptides (2). Various peptide inhibitors of integrin $\alpha_V\beta_3$ have been isolated as disintegrins from snake venom. A special case for an inhibitory peptide is the collagen IV fragment tumstatin because it acts as an endogenous regulator. Chemical peptide mimetics (3) are also available for the inhibition of integrin $\alpha_V\beta_3$ ligation. In contrast, the heterodimeric structure of integrin receptors makes the use of soluble receptors more difficult than it would be in the case of single chain receptors.

1. The monoclonal anti-integrin $\alpha_V\beta_3$ antibody 17E6 is effective in preventing the growth of melanomas through direct action on the tumors [147]. Indirect effects may also contribute. Tumor-induced angiogenesis promotes vascular cell entry into the cell cycle and expression of integrin $\alpha_V\beta_3$. A single dose of monoclonal antibody (LM609) to this integrin induces apoptosis of the proliferative angiogenic blood vessel cells and leads to tumor regression. Tumors treated with LM609 contain significantly fewer blood vessels and are considerably less invasive than controls. This is also the case for tumors that do not express this integrin, suggesting that the effect is due to inhibition of the integrin receptors expressed on proliferating endothelial cells [25,148]. The intravenous administration of the chimeric antibody c7E3 Fab has been considered in coronary angioplasty to prevent smooth muscle cell adhesion on, and migration to, vitronectin and osteopontin. The combination of anti-integrin $\alpha_V\beta_5$ antibody and c7E3 Fab had a greater effect than either antibody alone. PDGF-induced smooth muscle cell migration was also inhibited by c7E3 Fab and LM609, but to a much lesser extent [149]. This antibody combination may also suppress angiogenesis in tumors.
2. A cyclic RGD peptide prevents the growth of melanomas through direct action on the tumors [147]. Vascular cells stimulated to enter the cell cycle undergo apoptosis in the presence of integrin $\alpha_V\beta_3$ antagonists, such as a cyclic peptide (203, RGDfV) but not with a control peptide (601, RADfV) [25]. In vivo screening of a phage peptide library for motifs that home to tumor vasculature, independent of tumor type, identified the sequence CDCRGDCFC, which binds selectively to integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$. When coupled to the anticancer agent doxorubicin, this peptide enhanced the efficacy of the drug suggesting that it is possible to target chemotherapy to the tumor vasculature [26].
   The collagen fragment tumstatin (α3(IV)NC1 domain, non-collagenous 1 domain of the α3 chain of type IV collagen) possesses C-terminal (amino acids 185–203) anti-tumor activity. The 185–203 amino acid sequence is a ligand for integrin $\alpha_V\beta_3$, which binds both endothelial cells and melanoma cells but only inhibits the proliferation of melanoma cells. Tumstatin (α3(IV)NC1 domain) also possesses an N-terminal (amino acids 54–132) anti-angiogenic activity. While this fragment binds both endothelial cells and melanoma cells, it inhibits the proliferation of endothelial cells with no

effect on tumor cell proliferation. The two distinct RGD-independent binding sites on tumstatin imply unique integrin $\alpha_V\beta_3$-mediated mechanisms governing its two distinct anti-tumor properties [150].

Several snake venoms inhibit platelet aggregation by blocking integrin receptors. Through the same mechanism, they may also inhibit cancer cell adhesion and invasion. Contortrostatin is a unique dimeric disintegrin isolated from southern copperhead snake venom, which antagonizes integrins $\alpha_5\beta_1$, $\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$, and $\alpha_V\beta_5$, and prevents the invasion of cancer cells through matrigel [151]. The snake venom echistatin inhibits integrin $\alpha_V\beta_3$-dependent osteoclastic bone resorption. The use of small, echistatin-derived peptide drugs has been suggested for the treatment of osteolytic bone metastases [152].

3. A synthetic chemical peptide mimetic, β-[2-[[5-[(aminoiminomethyl)amino]-1-oxopentyl]amino]-1-oxoethyl]amino-3-pyridinepropanoic acid, bistrifluoroacetate (SC-56631), recognizes integrins $\alpha_V\beta_3$, $\alpha_V\beta_5$, and less effectively $\alpha_V\beta_1$ [54] and may be suitable as cancer therapeutic. The compound can also block bone resorption and platelet β_3 integrins, however, no bleeding was observed in rats that were administered the drug for 6 weeks. The two synthetic peptidomimetic antagonists SC-56631 and SC-65811 inhibit osteoclast adhesion to osteopontin- and vitronectin-coated surfaces and induce rapid retraction of osteoclasts from immobilized osteopontin [153], a mechanism relevant to lytic bone metastases.

7.3. Inhibition of integrin $\alpha_V\beta_3$ associated signal transduction following engagement by osteopontin

Signal transduction through integrin receptors depends in part on cell morphology and may proceed in two phases (Fig. 2). Haptotaxis ('cell crawling') on osteopontin, mediated by integrin $\alpha_V\beta_3$, is dependent on PKC activity. Once the cell reaches the highest concentration of the ligand, it spreads and additional signal transduction pathways, associated with PI 3-kinase and MAP kinase, are activated (S. Ashkar and G.F. Weber, unpublished results). Integrin $\alpha_V\beta_3$-dependent cell spreading on osteopontin is mediated by two principal pathways. Firstly, the cascade integrin $\alpha_V\beta_3 \rightarrow$ p125 FAK/c-Src $\rightarrow$ PI 3-kinase, enhanced by a trimolecular complex of paxillin, FAK, and Csk, leads to rearrangement of actin and gelsolin. Secondly, the pathway FAK/c-Src $\rightarrow$ Grb $\rightarrow$ Sos $\rightarrow$ Ras, enhanced by FAK $\rightarrow$ paxillin $\rightarrow$ Crk $\rightarrow$ C3G $\rightarrow$ Ras, leads to MAP kinase activation, gene expression, and cytoskeletal changes [154–156].

As detailed in Section 4.2, inhibitors of PKC are under study as growth suppressors and they may downregulate osteopontin gene expression. Their inhibition of integrin $\alpha_V\beta_3$-mediated haptotaxis could represent an additional beneficial mechanism. Investigations into tumor spread should be included in the evaluation of the efficacy of PKC inhibitors in cancer treatment. PI 3-kinase and Src are proto-oncogenes that also provide suitable targets for tumor therapy. Their inhibition may curtail integrin-dependent mechanisms of tumor invasion.

8. Integrin β_1 receptors as therapeutic targets

The interaction between osteopontin and β_1 integrins is unusual insofar as it represents binding to an integrin independently of the RGD motif. Collagen type IV is a major component of the basal lamina of blood vessels. Soluble non-collagenous (NC1) domains of the α2(IV), α3(IV), and α6(IV) chains of human collagen type IV negatively regulate angiogenesis and tumor growth. These NC1 domains inhibit endothelial cell adhesion and migration by distinct α_V and β_1 integrin-dependent mechanisms. Systemic administration of recombinant α2(IV), α3(IV), and α6(IV) NC1 domains potently inhibits angiogenesis and tumor growth, whereas α1(IV), α4(IV), and α5(IV) show little, if any, effect [157].

9. Conclusions

In current clinical practice, suppression of tumor growth is the primary target of anti-cancer therapy. This causes undesired effects on other proliferating tissues, leading for example to hair loss and vomiting. In addition, DNA damaging anti-proliferative agents themselves have carcinogenic potential. These regimens also affect only a small fraction of molec-

Table 2
Synopsis of therapeutic potential of targeting osteopontin

Target	Drug	Potential	Challenge
Osteopontin induction			
Steroid pathway	Estrogens	Particularly suited for hormone-sensitive tumors	
PKC	Go6976		
	Safingol		
	Calphostin C		
	7-Hydroxystaurosporine		
	PKC412		
Ras	Hypothemycin		
	Ribozyme	High specificity	Drug delivery
Osteopontin message	Ribozyme	High specificity	Drug delivery
	Antisense	Multiple points of interaction at single receptor site, action before protein is synthesized, straightforward drug design	
Osteopontin protein	Antibody	High specificity, high affinity	Rapid inactivation in vivo
	Synthetic peptide	Cellular uptake is not necessary	RGD peptides are non-specific
	Engineered peptides	Latitude in design, introduction of additional functions	Potential antigenicity, limited control over adverse effects
Posttranslational modifications			Drugs in this category may influence multiple enzyme substrates
Thrombin	Thrombin inhibitors	Already in clinical use	Multiple effects on other systems, including coagulation
Protein kinase			
Transglutaminase	Monodansylcadaverine		
	Cystamine		
CD44			
Expression	E1A gene therapy		Drug delivery, toxicity
Ligation	Antibody to v6	Tumor killing by ADCC	Drug delivery, rapid inactivation in vivo
	Synthetic peptide		
	Soluble CD44		
Cytotoxic therapy	Antibody radioconjugate	High specificity, high affinity	
	Osteopontin cytotoxic conjugates	Uptake by receptor internalization	Untested
Immunotherapy	Bispecific antibody (CR3–CD44v6)	Direction of macrophages to the tumor	Lower affinity to both antigens, Fc-mediated cell killing less effective than with anti-CD44 antibody
	Engineered CTL (TCR ζ–single chain anti-CD44v6)	Enhanced tumor recognition and lysis	Dependent on ex vivo manipulation to CTLs, possibly insufficient to eradicate large tumors
	Engineered CTL (TCR ζ–single chain anti-CD44v7,8)		

Table 2 (*continued*)

Target	Drug	Potential	Challenge
Disruption of heparin linkage			
Integrin $\alpha_V\beta_3$			
Expression	Dexamethasone		Drug delivery
	Intracellular single chain antibody	High specificity	
Ligation	Antibody	High specificity, high affinity	Rapid inactivation in vivo
	Synthetic peptide	Cellular uptake is not necessary	RGD peptides are non-specific
	Peptide mimetics		
	Tumstatin		Toxicity
	Snake venom		
Signal transduction			
– PKC	(see above)		
– PI 3-kinase			
– Src			
Integrin β_1			Contribution of integrin β_1 chain to tumor metastasis is unclear
Ligation	Collagen IV fragments		

The table summarizes molecular connections associated with osteopontin functions and compounds that modulate them. Potential and challenges of these compounds for clinical development are outlined.

ular events that are necessary for the malignant transformation of cells, suggesting that therapeutic effectiveness could be improved by broadening the armamentarium to also combat immortalization and dissemination.

The molecular machinery of metastasis formation represents suitable targets for intervention. Because the molecules involved comprise receptors and soluble mediators, they are more readily reached by pharmaceuticals than intracellular drug targets. In addition, since metastasis genes are stress response genes, their expression in the adult healthy organism is very restricted so that unwanted effects associated with their inhibition may be limited.

While the identity of relevant metastasis molecules (homing receptors and their specific ligands) depends on the origin of the cancer, osteopontin and its receptors figure prominently in a wide spectrum of malignancies. Drugs that target osteopontin-dependent mechanisms could potentially support the clinical intervention against multiple tumors (Table 2). In this review, we set out to identify molecular processes, associated with osteopontin functions in cancer, which could be subject to therapeutic modulation. Some of the connections outlined appear specific and suitable as drug targets. This is the case for the ligation of integrin $\alpha_V\beta_3$, for which inhibitors are under study as lead anti-cancer compounds. In other cases, such as the reversal of the malignant phenotype by suppression of osteopontin RNA with antisense probes or ribozymes, experimental proof of principle has been obtained, but lead compounds for drug development are not yet available. Similarly, inhibition of several activated oncogene products, including Ras and Src, would curb not only cell cycle progression but also the induction of osteopontin. Suitable drug candidates are, however, sparse. Naturally, many components in the cascade of osteopontin-dependent migration and invasion of malignant cells do not represent promising targets for therapeutic intervention. They include the enzymes involved in posttranslational modifications and many signal transduction molecules, all of which contribute to a diverse array of biological functions, whose suppression is likely to be harmful. Further studies are needed to define osteopontin associated drug targets and to translate these insights into effective anti-cancer agents.

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References

[1] G.F. Weber, S. Ashkar, Stress response genes – the genes that make cancer metastasize, J. Mol. Med. 78 (2000) 404–408.
[2] R. Arch, K. Wirth, M. Hofmann, H. Ponta, S. Matzku, P. Herrlich, M. Zoller, Participation in normal immune responses of a metastasis-inducing splice variant of CD44, Science 257 (1992) 682–685.
[3] E.S. Sorensen, P. Hojrup, T.E. Petersen, Posttranslational modifications of bovine osteopontin: identification of twenty-eight phosphorylation and three O-glycosylation sites, Protein Sci. 4 (1995) 2040–2049.
[4] E.S. Sorensen, T.E. Petersen, Identification of two phosphorylation motifs in bovine osteopontin, Biochem. Biophys. Res. Commun. 198 (1994) 200–205.
[5] E. Salih, H.Y. Zhou, M.J. Glimcher, Phosphorylation of purified bovine bone sialoprotein and osteopontin by protein kinases, J. Biol. Chem. 271 (1996) 16897–16905.
[6] E. Salih, S. Ashkar, L.C. Gerstenfeld, M.J. Glimcher, Identification of the phosphorylated sites of metabolically 32P-labeled osteopontin from cultured chicken osteoblasts, J. Biol. Chem. 272 (1997) 13966–13973.
[7] R.P. Singh, R. Patarca, J. Schwartz, P. Singh, H. Cantor, Definition of a specific interaction between the early T lymphocyte activation 1 (Eta-1) protein and murine macrophages in vitro and its effect upon macrophages in vivo, J. Exp. Med. 171 (1990) 1931–1942.
[8] D.T. Denhardt, A.F. Chambers, Overcoming obstacles to metastasis-defenses against host defenses: osteopontin (OPN) as a shield against attack by cytotoxic host cells, J. Cell Biochem. 56 (1994) 48–51.
[9] G.F. Weber, S. Ashkar, M.J. Glimcher, H. Cantor, Receptor-ligand interaction between CD44 and osteopontin (Eta-1), Science 271 (1996) 509–512.
[10] D.R. Senger, C.A. Perruzzi, Cell migration promoted by a potent GRGDS-containing thrombin-cleavage fragment of osteopontin, Biochim. Biophys. Acta 1314 (1996) 13–24.
[11] S. Ashkar, G.F. Weber, V. Panoutsakopoulou, M.E. Sanchirico, M. Janssen, S. Zawaideh, S. Rittling, D.T. Denhardt, M.J. Glimcher, H. Cantor, The cytokine Eta-1/Opn is an essential molecular bridge to type 1 (cell-mediated) immunity, Science 287 (2000) 860–864.
[12] R. Patarca, G.J. Freeman, R.P. Singh, F.Y. Wei, T. Durfee, F. Blattner, D.C. Regnier, C.A. Kozak, B.A. Mock, H.C. Morse III et al., Structural and functional studies of the early T lymphocyte activation 1 (Eta-1) gene. Definition of a novel T cell-dependent response associated with genetic resistance to bacterial infection, J. Exp. Med. 170 (1989) 145–161.
[13] G.J. Nau, P. Guilfoile, G.L. Chupp, J.S. Berman, S.J. Kim, H. Kornfeld, R.A. Young, A chemoattractant cytokine associated with granulomas in tuberculosis and silicosis, Proc. Natl. Acad. Sci. USA 94 (1997) 6414–6419.
[14] G.J. Nau, G.L. Chupp, J.F. Emile, E. Jouanguy, J.S. Berman, J.L. Casanova, R.A. Young, Osteopontin expression correlates with clinical outcome in patients with mycobacterial infection, Am. J. Pathol. 157 (2000) 37–42.
[15] G.J. Nau, L. Liaw, G.L. Chupp, J.S. Berman, B.L. Hogan, R.A. Young, Attenuated host resistance against *Mycobacterium bovis* BCG infection in mice lacking osteopontin, Infect. Immun. 67 (1999) 4223–4230.
[16] L. Liaw, D.E. Birk, C.B. Ballas, J.S. Whitsitt, J.M. Davidson, B.L. Hogan, Altered wound healing in mice lacking a functional osteopontin gene (spp1), J. Clin. Invest. 101 (1998) 1468–1478.
[17] F. Prols, B. Loser, M. Marx, Differential expression of osteopontin, PC4, and CEC5, a novel mRNA species, during in vitro angiogenesis, Exp. Cell Res. 239 (1998) 1–10.
[18] J. Wiener, D.M. Lombardi, J.E. Su, S.M. Schwartz, Immunohistochemical and molecular characterization of the differential response of the rat mesenteric microvasculature to angiotensin-II infusion, J. Vasc. Res. 33 (1996) 195–208.
[19] D. deBlois, D.M. Lombardi, E.J. Su, A.W. Clowes, S.M. Schwartz, C.M. Giachelli, Angiotensin II induction of osteopontin expression and DNA replication in rat arteries, Hypertension 28 (1996) 1055–1063.
[20] C.A. Henke, U. Roongta, D.J. Mickelson, J.R. Knutson, J.B. McCarthy, CD44-related chondroitin sulfate proteoglycan, a cell surface receptor implicated with tumor cell invasion, mediates endothelial cell migration on fibrinogen and invasion into a fibrin matrix, J. Clin. Invest. 97 (1996) 2541–2552.
[21] V. Trochon, C. Mabilat, P. Bertrand, Y. Legrand, F. Smadja-Joffe, C. Soria, B. Delpech, H. Lu, Evidence of involvement of CD44 in endothelial cell proliferation, migration and angiogenesis in vitro, Int. J. Cancer 66 (1996) 664–668.
[22] L. Liaw, M.P. Skinner, E.W. Raines, R. Ross, D.A. Cheresh, S.M. Schwartz, C.M. Giachelli, The adhesive and migratory effects of osteopontin are mediated via distinct cell surface integrins. Role of alpha v beta 3 in smooth muscle cell migration to osteopontin in vitro, J. Clin. Invest. 95 (1995) 713–724.
[23] D.R. Senger, S.R. Ledbetter, K.P. Claffey, A. Papadopoulos-Sergiou, C.A. Peruzzi, M. Detmar, Stimulation of endothelial cell migration by vascular permeability factor/vascular endothelial growth factor through cooperative mechanisms involving the alphavbeta3 integrin, osteopontin, and thrombin, Am. J. Pathol. 149 (1996) 293–305.
[24] M. Scatena, M. Almeida, M.L. Chaisson, N. Fausto, R.F. Nicosia, C.M. Giachelli, NF-kappaB mediates alphavbeta3

integrin-induced endothelial cell survival, J. Cell Biol. 141 (1998) 1083–1093.
[25] P.C. Brooks, A.M.P. Montgomery, M. Rosenfeld, R.A. Reisfeld, T. Hu, G. Klier, D.A. Cheresh, Integrin $\alpha_v\beta_3$ antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels, Cell 79 (1994) 1157–1164.
[26] W. Arap, R. Pasqualini, E. Ruoslahti, Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model, Science 279 (1998) 377–380.
[27] G.F. Weber, B. Adler, S. Ashkar, Osteopontin in oxidative stress responses, in: R.R. Ruffolo, Jr., G.Z. Feuerstein, A.J. Hunter, G. Poste, B.W. Metcalf (Eds.), Inflammatory Cells and Mediators in CNS Diseases, Harwood Academic Amsterdam, 1999, pp. 97–112.
[28] G.N. Thalmann, R.A. Sikes, R.E. Devoll, J.A. Kiefer, R. Markwalder, I. Klima, C.M. Farach-Carson, U.E. Studer, L.W. Chung, Osteopontin: possible role in prostate cancer progression, Clin. Cancer Res. 5 (1999) 2271–2277.
[29] D.T. Denhardt, C.A. Lopez, E.E. Rollo, S.M. Hwang, X.R. An, S.E. Walther, Osteopontin-induced modifications of cellular functions, Ann. NY Acad. Sci. 760 (1995) 127–142.
[30] J.G. Kleinman, E.M. Worcester, A.M. Beshensky, A.M. Sheridan, J.V. Bonventre, D. Brown, Upregulation of osteopontin expression by ischemia in rat kidney, Ann. NY Acad. Sci. 760 (1995) 321–323.
[31] B.J. Padanilam, D.R. Martin, M.R. Hammerman, Insulin-like growth factor I-enhanced renal expression of osteopontin after acute ischemic injury in rats, Endocrinology 137 (1996) 2133–2140.
[32] B. Adler, S. Ashkar, H. Cantor, G.F. Weber, Costimulation through adhesion molecules determines the response to ligation of the T-cell antigen receptor, Cell. Immunol. 210 (2001) 30–40.
[33] J. Noti, Adherence to osteopontin via alphavbeta3 suppresses phorbol ester-mediated apoptosis in MCF-7 breast cancer cells that overexpress protein kinase C-alpha, Int. J. Oncol. 17 (2000) 1237–1243.
[34] D.S. Bautista, J-w. Xuan, C. Hota, A.F. Chambers, J.F. Harris, Inhibition of Arg-Gly-Asp (RGD)-mediated cell adhesion to osteopontin by a monoclonal antibody against osteopontin, J. Biol. Chem. 269 (1994) 23280–23285.
[35] D.R. Senger, C.A. Perruzzi, A. Papadopoulos-Sergiou, L. Van de Water, Adhesive properties of osteopontin: regulation by a naturally occurring thrombin-cleavage in close proximity to the GRGDS cell-binding domain, Mol. Biol. Cell 5 (1994) 565–574.
[36] C.W. Prince, D. Dickie, C.L. Krumdieck, Osteopontin, a substrate for transglutaminase and factor XIII activity, Biochem. Biophys. Res. Commun. 177 (1991) 1205–1210.
[37] S. Beninati, D.R. Senger, E. Cordella-Miele, A.B. Mukherjee, I. Chackalaparampil, V. Shanmugam, K. Singh, B.B. Mukherjee, Osteopontin its transglutaminase-catalyzed post-translational modifications and cross-linking to fibronectin, J. Biochem. (Tokyo) 115 (1994) 675–682.
[38] E.S. Sorensen, L.K. Rasmussen, L. Moller, P.H. Jensen, P. Hojrup, T.E. Petersen, Localization of transglutaminase-reactive glutamine residues in bovine osteopontin, Biochem. J. 304 (Pt 1) (1994) 13–16.
[39] D. Aeschlimann, D. Mosher, M. Paulsson, Tissue transglutaminase and factor XIII in cartilage and bone remodeling, Semin. Thromb. Hemost. 22 (1996) 437–443.
[40] T. Nagata, R. Todescan, H.A. Goldberg, Q. Zhang, J. Sodek, Sulphation of secreted phosphoprotein 1 (SPP1, osteopontin) is associated with mineralized tissue formation, Biochem. Biophys. Res. Commun. 165 (1989) 234–240.
[41] D.R. Senger, B.B. Asch, B.D. Smith, C.A. Perruzzi, H.F. Dvorak, A secreted phosphoprotein marker for neoplastic transformation of both epithelial and fibroblastic cells, Nature 302 (1983) 714–715.
[42] D.R. Senger, C.A. Perruzzi, Secreted phosphoprotein markers for neoplastic transformation of human epithelial and fibroblastic cells, Cancer Res. 45 (11 Pt 2) (1985) 5818–5823.
[43] A.F. Chambers, E.I. Behrend, S.M. Wilson, D.T. Denhardt, Induction of expression of osteopontin (OPN; secreted phosphoprotein) in metastatic, ras-transformed NIH 3T3 cells, Anticancer Res. 12 (1992) 43–47.
[44] R. Barraclough, H.J. Chen, B.R. Davies, M.P. Davies, Y. Ke, B.H. Lloyd, A. Oates, P.S. Rudland, Use of DNA transfer in the induction of metastasis in experimental mammary systems, Biochem. Soc. Symp. 63 (1998) 273–294.
[45] D.T. Denhardt, X. Guo, Osteopontin: a protein with diverse functions, FASEB J. 7 (1993) 1475–1482.
[46] E.I. Behrend, A.M. Craig, S.M. Wilson, D.T. Denhardt, A.F. Chambers, Reduced malignancy of ras-transformed NIH 3T3 cells expressing antisense osteopontin RNA, Cancer Res. 54 (1994) 832–837.
[47] H.A. Gardner, B. Berse, D.R. Senger, Specific reduction in osteopontin synthesis by antisense RNA inhibits the tumorigenicity of transformed Rat1 fibroblasts, Oncogene 9 (1994) 2321–2326.
[48] Y. Wu, D.T. Denhardt, S.R. Rittling, Osteopontin is required for full expression of the transformed phenotype by the ras oncogene, Br. J. Cancer 83 (2000) 156–163.
[49] M.S. Sy, D. Liu, R. Schiavone, J. Ma, H. Mori, Y. Guo, Interactions between CD44 and hyaluronic acid: their role in tumor growth and metastasis, Curr. Top. Microbiol. Immunol. 213 (Pt 3) (1996) 129–153.
[50] U. Guenthert, M. Hofmann, W. Rudy, S. Reber, M. Zoeller, I. Haussmann, S. Matzku, A. Wenzel, H. Ponta, P. Herrlich, A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells, Cell 65 (1990) 13–24.
[51] H. Liapis, A. Flath, S. Kitazawa, Integrin alpha V beta 3 expression by bone-residing breast cancer metastases, Diagn. Mol. Pathol. 5 (1996) 127–135.
[52] R. Max, R.R. Gerritsen, P.T. Nooijen, S.L. Goodman, A. Sutter, U. Keilholz, D.J. Ruiter, R.M. De Waal, Immunohistochemical analysis of integrin alpha vbeta3 expression on tumor-associated vessels of human carcinomas, Int. J. Cancer 71 (1997) 320–324.
[53] K.J. Bayless, R. Salazar, G.E. Davis, RGD-dependent va-

cuolation and lumen formation observed during endothelial cell morphogenesis in three-dimensional fibrin matrices involves the alpha(v)beta(3) and alpha(5)beta(1) integrins, Am. J. Pathol. 156 (2000) 1673–1683.

[54] V.W. Engleman, G.A. Nickols, F.P. Ross, M.A. Horton, D.W. Griggs, S.L. Settle, P.G. Ruminski, S.L. Teitelbaum, A peptidomimetic antagonist of the $\alpha_v\beta_3$ integrin inhibits bone resorption in vitro and prevents osteoporosis in vivo, J. Clin. Invest. 99 (1997) 2284–2292.

[55] D.D. Hu, E.C. Lin, N.L. Kovach, J.R. Hoyer, J.W. Smith, A biochemical characterization of the binding of osteopontin to integrins alpha v beta 1 and alpha v beta 5, J. Biol. Chem. 270 (1995) 26232–26238.

[56] S.T. Barry, S.B. Ludbrook, E. Murrison, C.M. Horgan, A regulated interaction between $\alpha_5\beta_1$ integrin and osteopontin, Biochem. Biophys. Res. Commun. 267 (2000) 764–769.

[57] P.C. Brooks, R.L. Klemke, S. Schon, J.M. Lewis, M.A. Schwartz, D.A. Cheresh, Insulin-like growth factor receptor cooperates with integrin $\alpha_v\beta_5$ to promote tumor cell dissemination in vivo, J. Clin. Invest. 99 (1997) 1390–1398.

[58] Y. Yokosaki, N. Matsuura, T. Sasaki, I. Murakami, H. Schneider, S. Higashiyama, Y. Saitoh, M. Yamakido, Y. Taooka, D. Sheppard, The integrin alpha(9)beta(1) binds to a novel recognition sequence (SVVYGLR) in the thrombin-cleaved amino-terminal fragment of osteopontin, J. Biol. Chem. 274 (1999) 36328–36334.

[59] S.T. Barry, S.B. Ludbrook, E. Murrison, C.M. Horgan, Analysis of the alpha4beta1 integrin-osteopontin interaction, Exp. Cell Res. 258 (2000) 342–351.

[60] K.J. Bayless, G.A. Meininger, J.M. Scholtz, G.E. Davis, Osteopontin is a ligand for the alpha4beta1 integrin, J. Cell Sci. 111 (1998) 1165–1174.

[61] Y.U. Katagiri, J. Sleeman, H. Fujii, P. Herrlich, H. Hotta, K. Tanaka, S. Chikuma, H. Yagita, K. Okumura, M. Murakami, I. Saiki, A.F. Chambers, T. Uede, CD44 variants but not CD44s cooperate with beta1-containing integrins to permit cells to bind to osteopontin independently of arginine-glycine-aspartic acid, thereby stimulating cell motility and chemotaxis, Cancer Res. 59 (1999) 219–226.

[62] M. Noda, K. Yoon, C.W. Prince, W.T. Butler, G.A. Rodan, Transcriptional regulation of osteopontin production in rat osteosarcoma cells by type β transforming growth factor, J. Biol. Chem. 263 (1988) 13916–13921.

[63] X. Shi, S. Bai, L. Li, X. Cao, Hoxa-9 represses TGF-beta-induced osteopontin gene transcription, J. Biol. Chem. 276 (2001) 850–855.

[64] K.B. Atkins, R.U. Simpson, M.J. Somerman, Stimulation of osteopontin mRNA expression in HL-60 cells is independent of differentiation, Arch. Biochem. Biophys. 343 (1997) 157–163.

[65] I. Chackalaparampil, A. Peri, M. Nemir, M.D. Mckee, P.H. Lin, B.B. Mukherjee, A.B. Mukherjee, Cells in vivo and in vitro from osteopetrotic mice homozygous for c-src disruption show suppression of synthesis of osteopontin, a multifunctional extracellular matrix protein, Oncogene 12 (1996) 1457–1467.

[66] K.T. Tang, C. Capparelli, J.L. Stein, G.S. Stein, J.B. Lian, A.C. Huber, L.E. Braverman, W.J. DeVito, Acidic fibroblast growth factor inhibits osteoblast differentiation in vitro: altered expression of collagenase, cell growth-related, and mineralization-associated genes, J. Cell Biochem. 61 (1996) 152–166.

[67] S. Iseki, A.O. Wilkie, G.M. Morriss-Kay, Fgfr1 and Fgfr2 have distinct differentiation- and proliferation-related roles in the developing mouse skull vault, Development 126 (1999) 5611–5620.

[68] S. Iseki, A.O. Wilkie, J.K. Heath, T. Ishimaru, K. Eto, G.M. Morriss-Kay, Fgfr2 and osteopontin domains in the developing skull vault are mutually exclusive and can be altered by locally applied FGF2, Development 124 (1997) 3375–3384.

[69] S.B. Rodan, G. Wesolowski, K. Yoon, G.A. Rodan, Opposing effects of fibroblast growth factor and pertussis toxin on alkaline phosphatase, osteopontin, osteocalcin, and type I collagen mRNA levels in ROS 17/2.8 cells, J. Biol. Chem. 264 (1989) 19934–19941.

[70] H. Tanaka, H. Ogasa, J. Barnes, C.T. Liang, Actions of bFGF on mitogenic activity and lineage expression in rat osteoprogenitor cells: effect of age, Mol. Cell. Endocrinol. 150 (1999) 1–10.

[71] M. Goppelt-Struebe, T. Wiedemann, J. Heusinger-Ribeiro, M. Vucadinovic, M. Rehm, F. Prols, Cox-2 and osteopontin in cocultured platelets and mesangial cells: role of glucocorticoids, Kidney Int. 57 (2000) 2229–2238.

[72] K. Singh, J.L. Balligand, T.A. Fischer, T.W. Smith, R.A. Kelly, Glucocorticoids increase osteopontin expression in cardiac myocytes and microvascular endothelial cells, Role in regulation of inducible nitric oxide synthase, J. Biol. Chem. 270 (1995) 28471–28478.

[73] A. Omigbodun, P. Ziolkiewicz, C. Tessler, J.R. Hoyer, C. Coutifaris, Progesterone regulates osteopontin expression in human trophoblasts: a model of paracrine control in the placenta?, Endocrinology 138 (1997) 4308–4315.

[74] J.M. Vanacker, C. Delmarre, X. Guo, V. Laudet, Activation of the osteopontin promoter by the orphan nuclear receptor estrogen receptor related alpha, Cell Growth Differ. 9 (1998) 1007–1014.

[75] J.M. Vanacker, K. Pettersson, J.A. Gustafsson, V. Laudet, Transcriptional targets shared by estrogen receptor-related receptors (ERRs) and estrogen receptor (ER) alpha, but not by ERbeta, EMBO J. 18 (1999) 4270–4279.

[76] A. Krook, M.J. Rapoport, S. Anderson, H. Pross, Y.C. Zhou, D.T. Denhardt, T.L. Delovitch, T. Haliotis, p21ras and protein kinase C function in distinct and interdependent signaling pathways in C3H 10T1/2 fibroblasts, Mol. Cell. Biol. 13 (1993) 1471–1479.

[77] C.D. Toma, S. Ashkar, M.L. Gray, J.L. Schaffer, L.C. Gerstenfeld, Signal transduction of mechanical stimuli is dependent on microfilament integrity: identification of osteopontin

as a mechanically induced gene in osteoblasts, J. Bone Miner. Res. 12 (1997) 1626–1636.
[78] M. Takemoto, K. Yokote, M. Yamazaki, A.L. Ridall, W.T. Butler, T. Matsumoto, K. Tamura, Y. Saito, S. Mori, Enhanced expression of osteopontin by high glucose in cultured rat aortic smooth muscle cells, Biochem. Biophys. Res. Commun. 258 (1999) 722–726.
[79] M.A. Tucker, P.L. Chang, C.W. Prince, G.Y. Gillespie, T.B. Mapstone, TPA-mediated regulation of osteopontin in human malignant glioma cells, Anticancer Res. 18 (2A) (1998) 807–812.
[80] A.M. Craig, J.H. Smith, D.T. Denhardt, Osteopontin, a transformation-associated cell adhesion phosphoprotein, is induced by 12-O-tetradecanoylphorbol 13-acetate in mouse epidermis, J. Biol. Chem. 264 (1989) 9682–9689.
[81] R. Patarca, R.A. Saavedra, H. Cantor, Molecular and cellular basis of genetic resistance to bacterial infection: The role of the early T-lymphocyte activation-1/osteopontin gene, Crit. Rev. Immunol. 13 (1993) 225–246.
[82] A.F. Chambers, A.B. Tuck, Ras-responsive genes and tumor metastasis, Crit. Rev. Oncogen. 4 (1993) 95–114.
[83] Z.Z. Su, V.N. Austin, S.G. Zimmer, P.B. Fisher, Defining the critical gene expression changes associated with expression and suppression of the tumorigenic and metastatic phenotype in Ha-ras-transformed cloned rat embryo fibroblast cells, Oncogene 8 (1993) 1211–1219.
[84] X. Guo, Y.P. Zhang, D.A. Mitchell, D.T. Denhardt, A.F. Chambers, Identification of a ras-activated enhancer in the mouse osteopontin promoter and its interaction with a putative ETS-related transcription factor whose activity correlates with the metastatic potential of the cell, Mol. Cell. Biol. 15 (1995) 476–487.
[85] H. Tanaka, K. Nishida, K. Sugita, T. Yoshioka, Antitumor efficacy of hypothemycin, a new Ras-signaling inhibitor, Jpn. J. Cancer Res. 90 (1999) 1139–1145.
[86] T. Tokunaga, T. Tsuchida, H. Kijima, K. Okamoto, Y. Oshika, N. Sawa, Y. Ohnishi, H. Yamazaki, S. Miura, Y. Ueyama, M. Nakamura, Ribozyme-mediated inactivation of mutant K-ras oncogene in a colon cancer cell line, Br. J. Cancer 83 (2000) 833–839.
[87] M. Sato, E. Morii, T. Komori, H. Kawahata, M. Sugimoto, K. Terai, H. Shimizu, T. Yasui, H. Ogihara, N. Yasui, T. Ochi, Y. Kitamura, Y. Ito, S. Nomura, Transcriptional regulation of osteopontin gene in vivo by PEBP2alphaA/CBFA1 and ETS1 in the skeletal tissues, Oncogene 17 (1998) 1517–1525.
[88] Ki. Tezuka, D.T. Denhardt, G.A. Rodan, Si. Harada, Struimulation of mouse osteopontin promoter by v-Src is mediated by a CCAAT box-binding factor, J. Biol. Chem. 271 (1996) 22713–22717.
[89] V. Botquin, H. Hess, G. Fuhrmann, C. Anastassiadis, M.K. Gross, G. Vriend, H.R. Scholer, New POU dimer configuration mediates antagonistic control of an osteopontin preimplantation enhancer by Oct-4 and Sox-2, Genes Dev. 12 (1998) 2073–2090.
[90] D. Wang, S. Yamamoto, N. Hijiya, E.N. Benveniste, C.L. Gladson, Transcriptional regulation of the human osteopontin promoter functional analysis and DNA-protein interactions, Oncogene 19 (2000) 5801–5809.
[91] S. Kon, M. Maeda, T. Segawa, Y. Hagiwara, Y. Horikoshi, S. Chikuma, K. Tanaka, M.M. Rashid, M. Inobe, A.F. Chambers, T. Uede, Antibodies to different peptides in osteopontin reveal complexities in the various secreted forms, J. Cell Biochem. 77 (2000) 487–498.
[92] H.C. Crawford, L.M. Matrisian, L. Liaw, Distinct roles of osteopontin in host defense activity and tumor survival during squamous cell carcinoma progression in vivo, Cancer Res. 58 (1998) 5206–5215.
[93] S. Kasugai, Q. Zhang, C.M. Overall, J.L. Wrana, W.T. Butler, J. Sodek, Differential regulation of the 55 and 44 kDa forms of secreted phosphoprotein 1 (SPP-1, osteopontin) in normal and transformed rat bone cells by osteotropic hormones, growth factors and a tumor promoter, Bone Miner. 13 (1991) 235–250.
[94] V. Shanmugam, I. Chackalaparampil, G.C. Kundu, A.B. Mukherjee, B.B. Mukherjee, Altered sialylation of osteopontin prevents its receptor-mediated binding on the surface of oncogenically transformed tsB77 cells, Biochemistry 36 (1997) 5729–5738.
[95] M.C. Kiefer, D.M. Bauer, P.J. Barr, The cDNA and derived amino acid sequence for human osteopontin, Nucleic Acids Res. 17 (1989) 3306.
[96] G.F. Weber, S. Ashkar, H. Cantor, Interaction between CD44 and osteopontin as a potential basis for metastasis formation, Proc. Assoc. Am. Physicians 109 (1997) 1–9.
[97] T. Funato, T. Ishii, M. Kambe, K.J. Scanlon, T. Sasaki, Anti-K-ras ribozyme induces growth inhibition and increased chemosensitivity in human colon cancer cells, Cancer Gene Ther. 7 (2000) 495–500.
[98] S.A. Gibson, C. Pellenz, R.E. Hutchison, F.R. Davey, E.J. Shillitoe, Induction of apoptosis in oral cancer cells by an anti-bcl-2 ribozyme delivered by an adenovirus vector, Clin. Cancer Res. 6 (2000) 213–222.
[99] M.E. Halatsch, U. Schmidt, I.C. Botefur, J.F. Holland, T. Ohnuma, Marked inhibition of glioblastoma target cell tumorigenicity in vitro by retrovirus-mediated transfer of a hairpin ribozyme against deletion-mutant epidermal growth factor receptor messenger RNA, J. Neurosurg. 92 (2000) 297–305.
[100] J.S. Norris, B. Hoel, D. Voeks, F. Maggouta, M. Dahm, W. Pan, G. Clawson, Design and testing of ribozymes for cancer gene therapy, Adv. Exp. Med. Biol. 465 (2000) 293–301.
[101] T. Suzuki, B. Anderegg, T. Ohkawa, A. Irie, O. Engebraaten, M. Halks-Miller, P.S. Holm, D.T. Curiel, M. Kashani-Sabet, K.J. Scanlon, Adenovirus-mediated ribozyme targeting of HER-2/neu inhibits in vivo growth of breast cancer cells, Gene Ther. 7 (2000) 241–248.
[102] T. Tsuchida, H. Kijima, S. Hori, Y. Oshika, T. Tokunaga, K. Kawai, H. Yamazaki, Y. Ueyama, K.J. Scanlon, N. Tamaoki, M. Nakamura, Adenovirus-mediated anti-K-ras ribozyme induces apoptosis and growth suppression of hu-

man pancreatic carcinoma, Cancer Gene Ther. 7 (2000) 373–383.
[103] B. Feng, E.E. Rollo, D.T. Denhardt, Osteopontin (OPN) may facilitate metastasis by protecting cells from macrophage NO-mediated cytotoxicity: evidence from cell lines down-regulated for OPN expression by targeted ribozyme, Clin. Exp. Metast. 13 (1995) 453–462.
[104] E.I. Behrend, A.M. Craig, S.M. Wilson, D.T. Denhardt, A.F. Chambers, Expression of antisense osteopontin RNA in metastatic mouse fibroblasts is associated with reduced malignancy, Ann. NY Acad. Sci. 760 (1995) 299–301.
[105] L. Su, A.B. Mukherjee, B.B. Mukherjee, Expression of antisense osteopontin RNA inhibits tumor promoter-induced neoplastic transformation of mouse JB6 epidermal cells, Oncogene 10 (1995) 2163–2169.
[106] P.D. Brown, Matrix metalloproteinase inhibitors, Breast Cancer Res. Treat. 52 (1998) 125–136.
[107] A.V. Schally, Hypothalamic hormones from neuroendocrinology to cancer therapy, Anticancer Drugs 5 (1994) 115–130.
[108] S.R. Burzynski, Antineoplastons: history of the research (I), Drugs Exp. Clin. Res. 12 (Suppl. 1) (1986) 1–9.
[109] S.R. Rittling, F. Feng, Detection of mouse osteopontin by western blotting, Biochem. Biophys. Res. Commun. 250 (1998) 287–292.
[110] M.H. Helfrich, S.A. Nesbitt, E.L. Dorey, M.A. Horton, Rat osteoclasts adhere to a wide range of RGD (Arg-Gly-Asp) peptide-containing proteins, including the bone sialoproteins and fibronectin, via a beta 3 integrin, J. Bone Miner. Res. 7 (1992) 335–343.
[111] A.F. Chambers, C. Hota, C.W. Prince, Adhesion of metastatic, ras-transformed NIH 3T3 cells to osteopontin, fibronectin, and laminin, Cancer Res. 53 (1993) 701–706.
[112] E. Pettersson, B. Luning, H. Mickos, D. Heinegard, Synthesis, NMR and function of an O-phosphorylated peptide, comprising the RGD-adhesion sequence of osteopontin, Acta Chem. Scand. 45 (1991) 604–608.
[113] H. Mickos, K. Sundberg, B. Luning, Synthesis, NMR spectra and function of peptides with alpha-methylserine attached to the RGD sequence of osteopontin, Acta Chem. Scand. 46 (1992) 989–993.
[114] A.G. Turpie, J.I. Weitz, J. Hirsh, Advances in antithrombotic therapy: novel agents, Thromb. Haemost. 74 (1995) 565–571.
[115] H.D. White, Clinical trials of direct thrombin inhibitors in acute ischaemic syndromes, Thromb. Haemost. 78 (1997) 364–366.
[116] J.K. French, H.D. White, Direct thrombin inhibitors as adjuncts to thrombolytic therapy, Curr. Cardiol. Rep. 1 (1999) 184–191.
[117] S. Ashkar, D.B. Teplow, M.J. Glimcher, R.A. Saavedra, In vitro phosphorylation of mouse osteopontin expressed in *E. coli*, Biochem. Biophys. Res. Commun. 191 (1993) 126–133.
[118] S. Ashkar, J.L. Schaffer, E. Salih, L.C. Gerstenfeld, M.J. Glimcher, Phosphorylation of osteopontin by Golgi kinases, Ann. NY Acad. Sci. 760 (1995) 296–298.
[119] E. Salih, S. Ashkar, L.C. Gerstenfeld, M.J. Glimcher, Protein kinases of cultured osteoblasts: selectivity for the extracellular matrix proteins of bone and their catalytic competence for osteopontin, J. Bone Miner. Res. 11 (1996) 1461–1473.
[120] M.T. Kaartinen, A. Pirhonen, A. Linnala-Kankkunen, P.H. Maenpaa, Transglutaminase-catalyzed cross-linking of osteopontin is inhibited by osteocalcin, J. Biol. Chem. 272 (1997) 22736–22741.
[121] F. Van Leuven, J.J. Cassiman, H. Van Den Berghe, Primary amines inhibit recycling of alpha 2M receptors in fibroblasts, Cell 20 (1980) 37–43.
[122] P.J. Birckbichler, G.R. Orr, M.K. Patterson, E. Conway, H.A. Carter, Increase in proliferative markers after inhibition of transglutaminase, Proc. Natl. Acad. Sci. USA 78 (1981) 5005–5008.
[123] M. Gilbert, W.J. Shaw, J.R. Long, K. Nelson, G.P. Drobny, C.M. Giachelli, P.S. Stayton, Chimeric peptides of statherin and osteopontin that bind hydroxyapatite and mediate cell adhesion, J. Biol. Chem. 275 (2000) 16213–16218.
[124] T.C. McDevitt, K.E. Nelson, P.S. Stayton, Constrained cell recognition peptides engineered into streptavidin, Biotechnol. Prog. 15 (1999) 391–396.
[125] H. Haegel-Kronenberger, H. delaSalle, A. Bohbot, J. Galon, E. Tartour, J.P. Cazenave, D. Hanau, Regulation of CD44 isoform expression and CD44-mediated signaling in human dendritic cells, Adv. Exp. Med. Biol. 417 (1997) 83–90.
[126] P. Kogerman, M.S. Sy, L.A. Culp, Oncogene-dependent expression of CD44 in Balb/c 3T3 derivatives: correlation with metastatic competence, Clin. Exp. Metast. 14 (1996) 73–82.
[127] M. Hofmann, W. Rudy, U. Gunthert, S.G. Zimmer, V. Zawadzki, M. Zoller, R.B. Lichtner, P. Herrlich, H. Ponta, A link between ras and metastatic behavior of tumor cells: ras induces CD44 promoter activity and leads to low-level expression of metastasis-specific variants of CD44 in CREF cells, Cancer Res. 53 (1993) 1516–1521.
[128] R. Bazell, Her-2: The Making of Herceptin, a Revolutionary Treatment for Breast Cancer, Random House, New York, 1998.
[129] S. Seiter, R. Arch, S. Reber, D. Komitowski, M. Hofmann, H. Ponta, P. Herrlich, S. Matzku, M. Zoeller, Prevention of tumor metastasis formation by anti-variant CD44, J. Exp. Med. 177 (1993) 443–455.
[130] T. Cook, W.P. Sheridan, Development of GnRH antagonists for prostate cancer new approaches to treatment, Oncologist 5 (2000) 162–168.
[131] A.G. Harris, Future medical prospects for Sandostatin, Metabolism 39 (9 Suppl. 2) (1990) 180–185.
[132] J. Cheng, T. Zhou, C. Liu, J.P. Shapiro, M.J. Brauer, M.C. Kiefer, P.J. Barr, J.D. Mountz, Protection from Fas-medi-

ated apoptosis by a soluble form of the Fas molecule, Science 263 (1994) 1759–1762.
[133] L.A. Rubin, D.L. Nelson, The soluble interleukin-2 receptor: biology, function, and clinical application, Ann. Intern. Med. 113 (1990) 619–627.
[134] N. Niitsu, K. Iijima, A. Chizuka, A high serum-soluble interleukin-2 receptor level is associated with a poor outcome of aggressive non-Hodgkin's lymphoma, Eur. J. Haematol. 66 (2001) 24–30.
[135] V. Bazil, V. Horejsi, Shedding of the CD44 adhesion molecule from leukocytes induced by anti-CD44 monoclonal antibody simulating the effect of a natural receptor ligand, J. Immunol. 149 (1992) 747–753.
[136] Y. Kawano, I. Okamoto, D. Murakami, H. Itoh, M. Yoshida, S. Ueda, H. Saya, Ras oncoprotein induces CD44 cleavage through phosphoinositide 3-OH kinase and the rho family of small G proteins, J. Biol. Chem. 275 (2000) 29628–29635.
[137] M.L. Alpaugh, M.C. Lee, M. Nguyen, M. Deato, L. Dishakjian, S.H. Barsky, Myoepithelial-specific CD44 shedding contributes to the anti-invasive and antiangiogenic phenotype of myoepithelial cells, Exp. Cell Res. 25 (2000) 150–158.
[138] R.M. Peterson, Q. Yu, I. Stamenkovic, B.P. Toole, Perturbation of hyaluronan interactions by soluble CD44 inhibits growth of murine mammary carcinoma cells in ascites, Am. J. Pathol. 156 (2000) 2159–2167.
[139] M.C. Lee, M.L. Alpaugh, M. Nguyen, M. Deato, L. Dishakjian, S.H. Barsky, Myoepithelial-specific CD44 shedding is mediated by a putative chymotrypsin-like sheddase, Biochem. Biophys. Res. Commun. 279 (2000) 116–123.
[140] K.H. Heider, M. Sproll, S. Susani, E. Patzelt, P. Beaumier, E. Ostermann, H. Ahorn, G.R. Adolf, Characterization of a high-affinity monoclonal antibody specific for CD44v6 as candidate for immunotherapy of squamous cell carcinomas, Cancer Immunol. Immunother. 43 (1996) 245–253.
[141] C. Somasundaram, R. Arch, S. Matzku, M. Zoller, Development of a bispecific F(ab')2 conjugate against the complement receptor CR3 of macrophages and a variant CD44 antigen of rat pancreatic adenocarcinoma for redirecting macrophage-mediated tumor cytotoxicity, Cancer Immunol. Immunother. 42 (1996) 343–350.
[142] U. Altenschmidt, D. Moritz, B. Groner, Specific cytotoxic T lymphocytes in gene therapy, J. Mol. Med. 4 (1997) 259–266.
[143] A. Hekele, P. Dall, D. Moritz, W. Wels, B. Groner, P. Herrlich, H. Ponta, Growth retardation of tumors by adoptive transfer of cytotoxic T lymphocytes reprogrammed by CD44v6-specific scFv:zeta-chimera, Int. J. Cancer 68 (1996) 232–238.
[144] P. Dall, A. Hekele, M.W. Beckmann, H.G. Bender, P. Herrlich, H. Ponta, Efficient lysis of CD44v7/8-presenting target cells by genetically engineered cytotoxic T-lymphocytes – a model for immunogene therapy of cervical cancer, Gynecol. Oncol. 66 (1997) 209–216.
[145] S.L. Cheng, C.D. Lai, A. Fausto, M. Chellaiah, X. Feng, K.P. McHugh, S.L. Teitelbaum, R. Civitelli, K.A. Hruska, F.P. Ross, L.V. Avioli, Regulation of alphaVbeta3 and alphaVbeta5 integrins by dexamethasone in normal human osteoblastic cells, J. Cell Biochem. 77 (2000) 265–276.
[146] P. Koistinen, T. Pulli, V.J. Uitto, L. Nissinen, T. Hyypia, J. Heino, Depletion of alphaV integrins from osteosarcoma cells by intracellular antibody expression induces bone differentiation marker genes and suppresses gelatinase (MMP-2) synthesis, Matrix Biol. 18 (1999) 239–251.
[147] F. Mitjans, T. Meyer, C. Fittschen, S. Goodman, A. Jonczyk, J.F. Marshall, G. Reyes, J. Piulats, In vivo therapy of malignant melanoma by means of antagonists of alphav integrins, Int. J. Cancer 87 (2000) 716–723.
[148] P.C. Brooks, S. Stromblad, R. Klemke, D. Visscher, F.H. Sarkar, D.A. Cheresh, Antiintegrin alpha v beta 3 blocks human breast cancer growth and angiogenesis in human skin, J. Clin. Invest. 96 (1995) 1815–1822.
[149] J.H. Baron, E.P. Moiseeva, D.P. de Bono, K.R. Abrams, A.H. Gershlick, Inhibition of vascular smooth muscle cell adhesion and migration by c7E3 Fab (abciximab): a possible mechanism for influencing restenosis, Cardiovasc. Res. 48 (2000) 464–472.
[150] Y. Maeshima, P.C. Colorado, R. Kalluri, Two RGD-independent alpha vbeta 3 integrin binding sites on tumstatin regulate distinct anti-tumor properties, J. Biol. Chem. 275 (2000) 23745–23750.
[151] Q. Zhou, P. Hu, M.R. Ritter, S.D. Swenson, S. Argounova, A.L. Epstein, F.S. Markland, Molecular cloning and functional expression of contortrostatin, a homodimeric disintegrin from southern copperhead snake venom, Arch. Biochem. Biophys. 375 (2000) 278–288.
[152] J.E. Fisher, M.P. Caulfield, M. Sato, H.A. Quartuccio, R.J. Gould, V.M. Garsky, G.A. Rodan, M. Rosenblatt, Inhibition of osteoclastic bone resorption in vivo by echistatin, an 'arginyl-glycyl-aspartyl' (RGD)-containing protein, Endocrinology 132 (1993) 1411–1413.
[153] C.P. Carron, D.M. Meyer, V.W. Engleman, J.G. Rico, P.G. Ruminski, R.L. Ornberg, W.F. Westlin, G.A. Nickols, Peptidomimetic antagonists of alphavbeta3 inhibit bone resorption by inhibiting osteoclast bone resorptive activity, not osteoclast adhesion to bone, J. Endocrinol. 165 (2000) 587–598.
[154] K.A. Hruska, F. Rolnick, M. Huskey, U. Alvarez, D. Cheresh, Engagement of the osteoclast integrin alpha v beta 3 by osteopontin stimulates phosphatidylinositol 3-hydroxyl kinase activity, Ann. NY Acad. Sci. 760 (1995) 151–165.
[155] M. Chellaiah, K. Hruska, Osteopontin stimulates gelsolin-associated phosphoinositide levels and phosphatidylinositol triphosphate-hydoxyl kinase, Mol. Biol. Cell 7 (1996) 743–753.

[156] M. Chellaiah, C. Fitzgerald, E.J. Filardo, D.A. Cheresh, K.A. Hruska, Osteopontin activation of c-src in human melanoma cells requires the cytoplasmic domain of the integrin alpha v-subunit, Endocrinology 137 (1996) 2432–2440.

[157] E. Petitclerc, A. Boutaud, A. Prestayko, J. Xu, Y. Sado, Y. Ninomiya, M.P. Sarras, B.G. Hudson, P.C. Brooks, New functions for non-collagenous domains of human collagen type IV. Novel integrin ligands inhibiting angiogenesis and tumor growth in vivo, J. Biol. Chem. 275 (2000) 8051–8061.

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Absence of the *CD44* Gene Prevents Sarcoma Metastasis[1]

Georg F. Weber,[2] Roderick T. Bronson, John Ilagan, Harvey Cantor, Rudolf Schmits,[3] and Tak W. Mak

Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, Boston, Massachusetts [G. F. W., J. I., H. C.]; Departments of Medicine [G. F. W.] and Pathology [J. I., H. C.], Harvard Medical School, Boston, Massachusetts 02115; USDA Human Nutrition Research Center on Aging, Tufts University, Boston, Massachusetts 02111 [R. T. B.]; and Department of Cellular and Molecular Biology, Ontario Cancer Institute, Toronto, Ontario M56 2M9, Canada [R. S., T. W. M.]

ABSTRACT

To test the role of the *CD44* gene in tumorigenesis, mice with the min mutation of the *APC* gene or with the tm1 mutation of the *p53* gene were crossed with CD44 knockout mice. The absence of *CD44* gene products did not affect tumor incidence or survival; however, mice with disruption of the *CD44* gene showed virtually aborted metastasis formation of osteosarcomas. This is in agreement with the role attributed to CD44 variants in the spread of cancer. Therefore, *CD44* gene products are not essential for tumor incidence and growth but are important in regulating metastasis formation.

INTRODUCTION

The transmembrane glycoprotein CD44 is expressed on lymphocytes and macrophages. It serves as a homing receptor that mediates binding to high endothelial venules and has also been implicated in lymphoid development. Lymphocyte activation results in the expression of multiple, alternatively spliced products of the *CD44* gene, which are generated by the insertion of ≤10 variant exons into the extracellular domain. In pathophysiology, aberrant expression of certain CD44 splice variants has been connected causally to the spread of diverse malignant cells (1, 2) and may distinguish metastasizing from nonmetastasizing tumors. This function is mediated by the cytokine ligand osteopontin (3). Additional roles in carcinogenesis have been attributed to various forms of CD44. Expression of this receptor on tumor cells may support tumor growth (4, 5), possibly after adhesion to hyaluronate, and signal transduction through CD44 can induce oncogenes, such as *ras* (6). In contrast, the standard form that lacks variant exons may exert suppression of tumor growth and dissemination (7). The contributions of these diverse CD44 functions to carcinogenesis are not fully elucidated.

Despite ample experimental evidence for a role of some forms of CD44 in malignancy, clinical studies relating expression of CD44 or its variants to prognosis in diverse cancers have remained controversial (8). Nonconclusive results in patients may have been obtained because of insufficient sensitivity or specificity of the analyses. Thus, the measurements of mRNA for specific CD44 variants expressed by tumors do not detect posttranslational modifications that may affect function (9). Furthermore, the CD44 receptor expressed on tumors represents one component of a functional pair. Specific ligands may bind to selective splice variants, so that the availability of these ligands contributes to determining the phenotype. Similarly, many conventional rodent models of malignancies are compromised because they rely on the injection of preformed tumor cells, often in nonphysiological locations, rather than on the generation of endogenous tumors. We set out to analyze the role of CD44 in a genetically defined and homogeneous system, which most closely resembles the pathophysiology of human cancers. Mouse models, where the role of individual genes is tested by breeding the relevant gene-targeted mice with mice that have high susceptibility to tumors because of mutations in tumor suppressor genes, have provided substantial insights. We used two endogenous tumor models using mice with point mutations in tumor suppressor genes with or without targeted deletion of the *CD44* gene: Mice with the $APC^{+/min}$ genotype display a predisposition to multiple intestinal neoplasia. The murine min mutation is a nonsense mutation, which is analogous to mutations found in human autosomal dominantly inherited familial adenomatous polyposis, as well as in sporadic colorectal cancers (10). $APC^{+/min}$ mice develop multiple benign intestinal tumors, whose growth reflects early steps of transformation. Mutations of the *p53* gene contribute to the pathogenesis of a large percentage of human cancers. Similarly, mice with one mutant allele of the *p53* gene are susceptible to a larger spectrum of tumors, predominantly sarcomas and lymphomas. These mice allow the investigation of malignant dissemination.

MATERIALS AND METHODS

Mice. Mice with point mutations in tumor suppressor genes, $APC^{+/min}$ bred on C57Bl/6 background or $trp53^{+/tm1}$ on C57Bl/6 background, were obtained from The Jackson Laboratory. Either $APC^{+/min}$ mice or $trp53^{+/tm1}$ mice were mated with $CD44^{-/-}$ mice that had been back-crossed from 129 to C57BL/6 for four generations (10). The genotypes were assessed using PCR from genomic DNA (10–12), and CD44 expression was confirmed by flow cytometry from blood samples using the pan-CD44 antibody 1M7 (PharMingen). Siblings were housed in groups of one to four per cage at the Redstone Animal Facility (DFCI) in alternate 12-h light and dark cycles. A diet of pelleted chow (Agway, Prolab 3000) and bottled water was administered *ad libitum*, and room temperature was kept at 25°C. The colony was tested frequently for endoparasitic and ectoparasitic infections, as well as for bacterial and viral infections by the Charles River Labs (Wilmington, MA). No infection was detected during the course of this study. Permission to exceed a tumor diameter of 2 cm was granted by the institutional animal care and use committee, and the mice were seen frequently by a veterinarian.

Inheritance. Mice with disrupted *CD44* genes were mated with heterozygotes for point mutations of the relevant tumor suppressor gene, yielding mice that were hemizygous for CD44 and either wild type or heterozygous for the tumor suppressor gene. Those two genotypes were interbred, which is expected to result in equal 12.5% representation of the genotypes of interest according to Mendelian inheritance (the remaining 2 × 25% are $CD44^{+/-}$). The litters from this second generation mating were screened. In the p53-related part of the study, 292 mice were analyzed, of which 16 were $trp53^{+/+}CD44^{+/+}$, 36 mice were $trp53^{+/+}CD44^{-/-}$, 24 mice had the genotype $trp53^{+/tm1}CD44^{+/+}$, and 26 mice had the genotype $trp53^{+/tm1}CD44^{-/-}$. In the APC-related part of the study, 217 mice were screened with the distribution of $APC^{+/+}CD44^{+/+}$ 21 mice, $APC^{+/+}CD44^{-/-}$ 20 mice, $APC^{+/min}CD44^{+/+}$ 10 mice, and $APC^{+/min}CD44^{-/-}$ 15 mice.

Because of the incomplete back-crossing from 129 to C57Bl/6, it is formally possible that a polymorphic modifier, linked to the CD44 locus, segregates with it and supersedes the influence of CD44 on tumor development. The tumor susceptibility locus Scc1 might be a candidate (13). This is unlikely, because quantitative trait loci, including Scc-1, depend strongly on interlocus

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[2] To whom requests for reprints should be addressed, at Department of Radiation Oncology, New England Medical Center and Tufts University Medical School, NEMC # 824, 750 Washington Street, Boston, MA 02111. Phone: (617) 636-9013; Fax: (617) 636-1766; E-mail: GWeber@lifespan.org.

[3] Present address: Universitaet des Saarlandes, 66424 Homburg, Germany.

interactions for influencing tumorigenesis (14). Cosegregation of one modifier could not affect the phenotype. It is generally improbable that genomic heterogeneity would account for the observations described here, because 97% of the genome are derived form the C57Bl/6 strain.

Necropsy. The animals were checked at least every 12 h, and total necropsy was performed on mice found moribund. Organs were fixed in formalin, and H&E slides were prepared for histological analysis. Histological evaluation was performed by an investigator blinded to the CD44 status of the samples.

Osteosarcoma metastases were observed in livers, lungs, and occasionally spleens. Enumeration of osteosarcoma metastases was performed on step sections from livers and lungs. For this purpose, every 10^{th} microtome cut corresponding to a step thickness of 60 μm was analyzed.

Immunohistochemistry. CD44 expression on osteosarcomas was assessed by immunohistochemistry with antibody KM114 after antigen retrieval with citrate. Staining was done with 3,3′-diaminobenzidine. Staining of all tumor samples with secondary antibody served as negative control, and a normal mouse spleen was used as a positive control (data not shown).

Enumeration of Intestinal Polyps. Entire intestines from stomach to rectum were extracted, washed in PBS, and fixed in 10% buffered formalin, and the number of polyps was counted under a dissection microscope. As controls, intestines from 3 $APC^{+/+}CD44^{+/+}$ mice at the ages of 420–442 days and 3 $APC^{+/+}CD44^{-/-}$ mice at the ages of 433–442 days were examined for spontaneous polyps.

Statistical Evaluation. The data sets were analyzed for statistically significant differences at 95% confidence by *t* test (after confirmation of normal distribution and equal variance) and by Wilcoxon Mann-Whitney test (after testing for equal distribution). The prerequisites for applicability of either test were not fulfilled for the metastasis data. They were analyzed for equal variance by the Cochran test.

RESULTS

Absence of CD44 Prevents Tumor Metastasis. Because aberrant expression of CD44 splice variants may confer a malignant phenotype to tumor cells, we asked whether the targeted deletion of the *CD44* gene was sufficient to suppress the dissemination of solid tumors. Osteosarcomas developed mostly on the lower back. One $trp53^{+/tm1}CD44^{-/-}$ mouse had an osteosarcoma of the skull. Metastases were detected in the lungs and livers from $trp53^{+/tm1}$ mice with osteosarcoma. Step sections from livers and lungs identified 28 metastases in 6 $CD44^{+/+}$ mice and 1 metastasis in 4 $CD44^{-/-}$ mice (Fig. 1). One $CD44^{+/+}$ mouse also displayed a macroscopically visible metastasis in the spleen. All 6 $CD44^{+/+}$ mice had multiple osteosarcoma metastases, whereas in 4 $CD44^{-/-}$ mice, only one individual lung metastasis was detected. Consistently, CD44 expression was prominent in the osteosarcomas of $CD44^{+/+}$ mice (Fig. 2).

Absence of CD44 Does Not Alter the Phenotype of Benign Tumors. The intestinal polyps caused by mutation in the *APC* gene grow noninvasively but express various splice variants of CD44. This occurs at the earliest stages of transformation diagnosed as aberrant crypt foci with dysplasia (15). Histology was performed on the largest intestinal polyps from each $APC^{+/min}$ mouse to assess malignancy. Consistent with previous reports, these tumors are noninvasive as judged by intact basement membranes in all cases (Fig. 3). No metastases were observed in other organs (three histological sections per organ). These results were not affected by the presence or absence of CD44. Histological findings included ectopic hepatic hematopoiesis and bone marrow

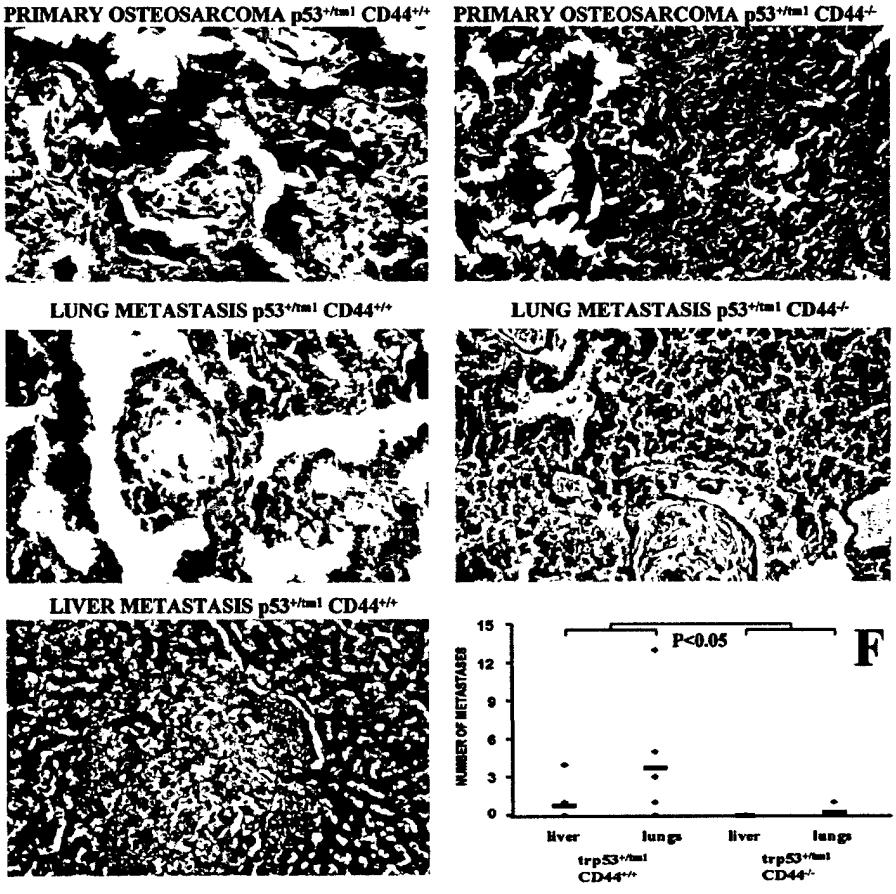

Fig. 1. Tumor invasiveness. *A–F*, osteosarcomas in $trp53^{+/tm1}$ mice and their metastases in lungs and liver. *A*, primary tumor in $CD44^{+/+}$ background; *B*, representative lung metastasis in $CD44^{+/+}$ background; *C*, representative liver metastasis in $CD44^{+/+}$ background; *D*, primary tumor in $CD44^{-/-}$ background; *E*, only metastasis detected in $CD44^{-/-}$ background (H&E); *F*, incidence of metastases in livers and lungs from osteosarcoma bearing mice.

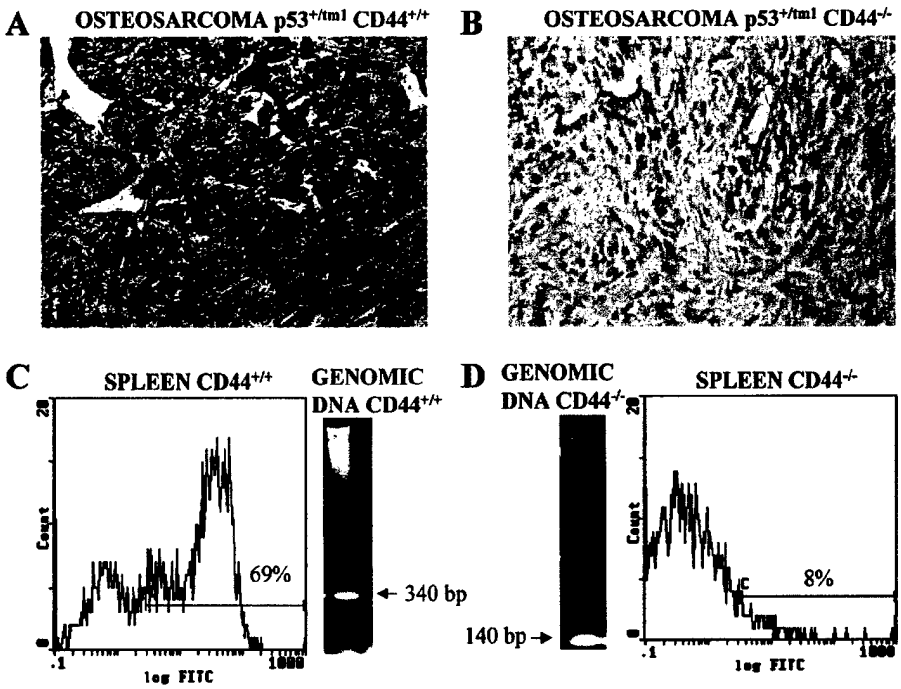

Fig. 2. Expression of CD44. *A* and *B*, immunohistochemistry for CD44 expression in osteosarcomas from a trp53$^{+/tm1}$ CD44$^{+/+}$ (*A*) and a trp53$^{+/tm1}$ CD44$^{-/-}$ mouse (*B*). In *C* and *D*, typing of the mice was performed by flow cytometry with FITC-anti-CD44 on spleen cells and lymph node cells (data not shown) and by PCR on genomic DNA with published primers (11); *C*, CD44$^{+/+}$; *D*, CD44$^{-/-}$.

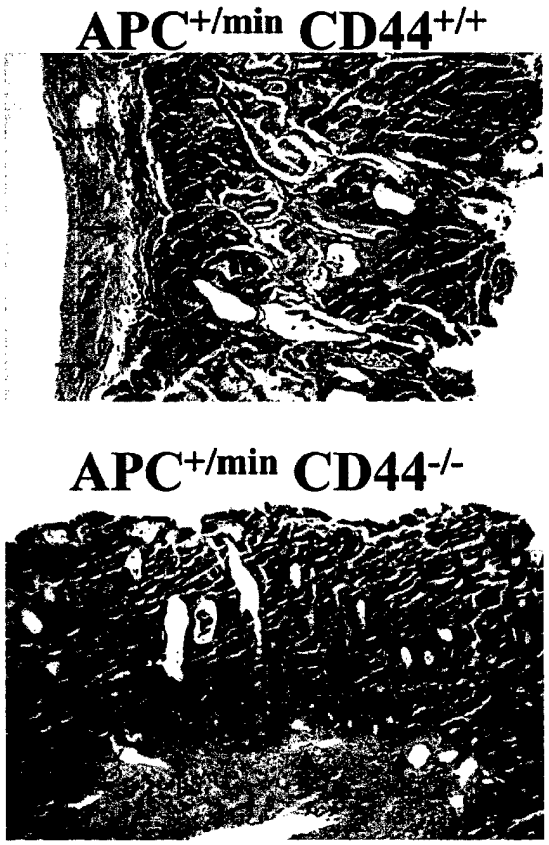

Fig. 3. Intestinal polyps caused by the min mutation of the *APC* gene are not invasive. Histology of representative intestinal polyps from APC$^{+/min}$ mice. The largest polyp from each intestine was sectioned in the middle and stained with H&E for histological assessment of signs for malignancy. Regardless of the presence of the *CD44* gene, the basement membrane remains intact (*arrows*). *A*, CD44$^{+/+}$; *B*, CD44$^{-/-}$.

siderosis in several mice, which are likely attributable to blood loss through the intestinal polyps.

CD44 Does Not Affect Tumor Incidence. We tested whether deletion of the *CD44* gene alters tumor incidence as judged by the number of intestinal polyps in mice with one mutated *APC* allele. All APC$^{+/min}$ mice succumbed to intestinal polyposis. At the time of death, APC$^{+/min}$ mice had developed around a mean of 66 polyps in CD44$^{+/+}$ background and 58 polyps in CD44$^{-/-}$ background (Fig. 4), suggesting that the tumor development in this model does not depend on the presence of *CD44* gene products.

Mice with the trp53$^{+/tm1}$ genotype developed, predominantly, sarcomas and lymphomas. The mesenchymal tumors were diagnosed as fibrosarcomas, osteosarcomas, hemangiosarcomas, and histiocytic sarcomas. Their incidence, associated life span, and tumor weight on death were not affected by the presence or absence of the *CD44* gene (Table 1). Like the osteosarcomas, the fibrosarcomas were located mostly on the lower back.

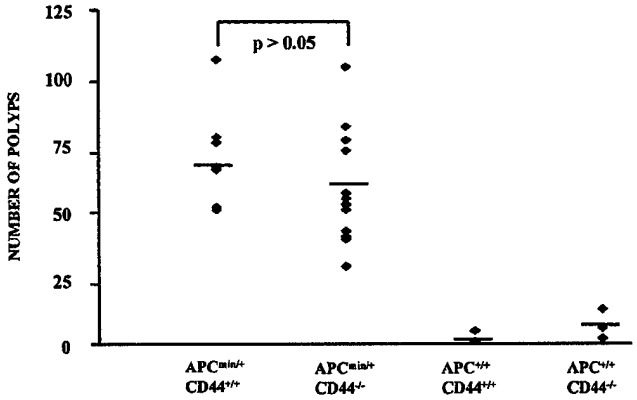

Fig. 4. Tumor incidence. The numbers of intestinal polyps were counted in APC$^{+/min}$ mice at the end of their life span (average 209 days for 10 APC$^{+/min}$CD44$^{+/+}$ mice and 236 days for 15 APC$^{+/min}$CD44$^{-/-}$ mice). The incidence of spontaneous polyps was assessed at an average of 433 days for 3 APC$^{+/+}$CD44$^{+/+}$ mice and 437 days for 3 APC$^{+/+}$CD44$^{-/-}$ mice. *Symbols*, individual data points; mean values are presented as *horizontal lines*.

Table 1 *Characterization of solid tumors in trp53$^{+/tm1}$ mice. Incidence, associated life span, and tumor weight at the time of death are specified for each histologic type of sarcoma for CD44$^{+/+}$ and CD44$^{-/-}$ genetic background. Life span and tumor weight are indicated as mean ± standard error.*

Tumor	Incidence	Life span		Weight	
Osteosarcoma					
trp53$^{tm1/+}$CD44$^{+/+}$	6 (25%) 5 f, 1 m[a]	532 ± 30 days	$P > 0.05$	7.7 ± 2.4 grams	$P > 0.05$
trp53$^{tm1/+}$CD44$^{-/-}$	4 (15%) 3 f, 1 m	467 ± 56 days		6.1 ± 1.7 grams	
Fibrosarcoma					
trp53$^{tm1/+}$CD44$^{+/+}$	7 (29%) 4 f, 3 m	410 ± 23 days	$P > 0.05$	21.9 ± 5.0 grams	$P > 0.05$
trp53$^{tm1/+}$CD44$^{-/-}$	7 (27%) 5 f, 2 m	403 ± 30 days		12.3 ± 2.9 grams	
Hemangiosarcoma					
trp53$^{tm1/+}$CD44$^{+/+}$	3 (12%) 2 f, 1 m	304 ± 51 days		10.7 ± 8.1 grams	
trp53$^{tm1/+}$CD44$^{-/-}$	0 (0%)				
trp53$^{+/+}$CD44$^{-/-}$	1 (3%) 1 m	595 days		2.9 grams	
Histiocytic sarcoma					
trp53$^{tm1/+}$CD44$^{+/+}$	2 (8%) 2 m	589/590 days			
trp53$^{tm1/+}$CD44$^{-/-}$	1 (4%) 1 f	420 days			
trp53$^{+/+}$CD44$^{+/+}$	1 (6%) 1 m	600 days			

[a] f = female; m = male.

Sporadic carcinomas also occurred in trp53$^{+/tm1}$ mice independently of their CD44 status with one case of squamous cell carcinoma in a trp53$^{+/tm1}$CD44$^{+/+}$ mouse and one incident of lung carcinoma among the trp53$^{+/tm1}$CD44$^{-/-}$ mice (Fig. 5).

There were four cases (17%) of lymphoma, typical of those observed in p53$^{+/tm1}$ mice (12, 16), in trp53$^{+/tm1}$CD44$^{+/+}$ mice with an associated mean life span of 445 days. In comparison, there were six cases (23%) of lymphomas, resembling anaplastic large cell lymphomas (17), in trp53$^{+/tm1}$CD44$^{-/-}$ mice with an associated life span of 503 days (data not shown). The morphology of the lymphoid malignancies in CD44$^{-/-}$ mice appeared unusual but requires further characterization.

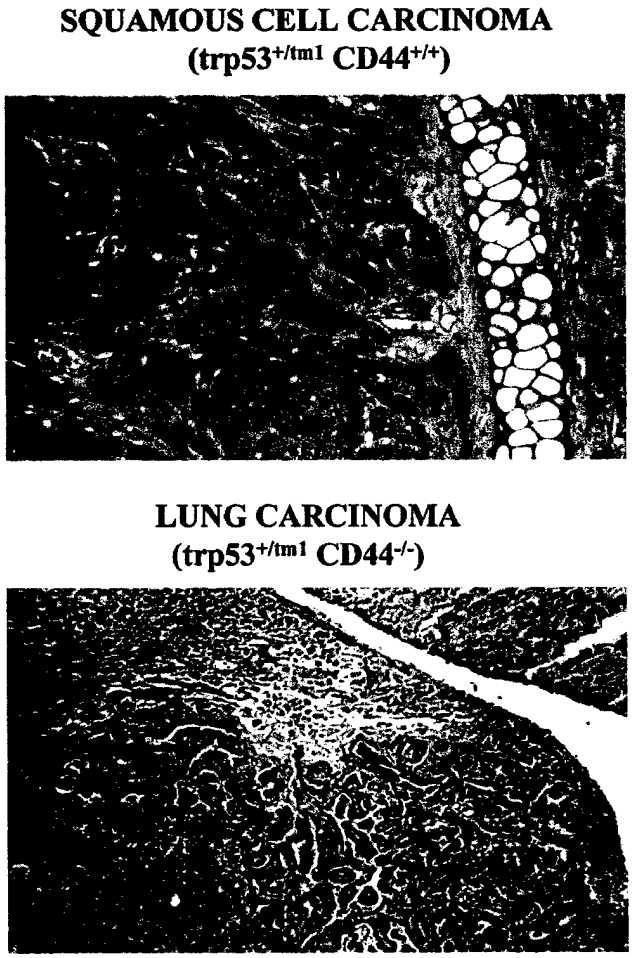

Fig. 5. Carcinomas in mice with one mutant *p53* allele. Histology of sporadic carcinomas in trp53$^{+/tm1}$ mice, including a squamous cell carcinoma (CD44$^{+/+}$; *top*) and a lung carcinoma (CD44$^{-/-}$; *bottom*). The slides are stained with H&E.

Five mice with one mutant *p53* allele had multiple tumors. In trp53$^{+/tm1}$CD44$^{+/+}$ mice, one osteosarcoma occurred together with a histiocytic sarcoma. Frequently, lymphomas were diagnosed in conjunction with solid tumors. One trp53$^{+/tm1}$CD44$^{+/+}$ mouse had lymphoma and osteosarcoma. In trp53$^{+/tm1}$CD44$^{-/-}$ mice, lymphoma was seen in conjunction with osteosarcoma, fibrosarcoma, and histiocytic sarcoma in one case each.

CD44 Does Not Affect Survival. The mice with one mutant allele of the *APC* gene developed symptoms of ruffled fur, bloated abdomen, and black stools followed by lethargy and succumbed around a mean of 209 days (range 133–350 days) of age, for CD44$^{+/+}$ background, or 236 days (range 96–326 days) of age, for CD44$^{-/-}$ background (Fig. 6*A*). At the end of their life span, 2 of the CD44$^{-/-}$ mice also suffered from rectal prolapse. None of the APC$^{+/+}$ mice died during the 420-day period of observation, regardless of their CD44 status.

Mice with one mutant *p53* allele developed various tumors with a predominance of sarcomas and lymphomas and had a 50% survival of ~470 days of age regardless of their CD44 genotype (Fig. 6*B*). The period of observation was limited to 600 days, at which point 1 of 24 trp53$^{+/tm1}$CD44$^{+/+}$ mice (4%) was still alive and 6 of 26 trp53$^{+/tm1}$CD44$^{-/-}$ mice (23%) survived. In the control groups, 14 of 16 trp53$^{+/+}$CD44$^{+/+}$ mice (87%) and 30 of 36 trp53$^{+/+}$CD44$^{-/-}$ mice (83%) were alive. Conversely, 1 trp53$^{+/tm1}$CD44$^{+/+}$ mouse (4%), 3 trp53$^{+/tm1}$CD44$^{-/-}$ mice (11%), 1 trp53$^{+/+}$CD44$^{+/+}$ mouse (6%), and 5 trp53$^{+/+}$CD44$^{-/-}$ mice (14%) died without detectable signs of malignancies. Although five of them were diagnosed with histological signs of inflammation (abscess, periarteriitis nodosa, glomerulonephritis, dermatitis, and pneumonia), the contribution of these conditions to the death of the mice is unknown. The higher incidence of deaths unrelated to neoplasms (8 CD44$^{-/-}$ mice of 50 when disregarding the trp53 status, compared with 2 CD44$^{+/+}$ mice of 40) implies that the lack of the *CD44* gene may increase the susceptibility to other pathogenic influences.

DISCUSSION

Diverse roles in cancer have been ascribed to various *CD44* gene products, but their contributions to endogenous tumors have not been studied. Here, we have tested the consequences of targeted deletion of the *CD44* gene in the development of endogenous tumors caused by mutations in two distinct tumor suppressor genes, which are also mutated frequently in human cancers. We show that the absence of the

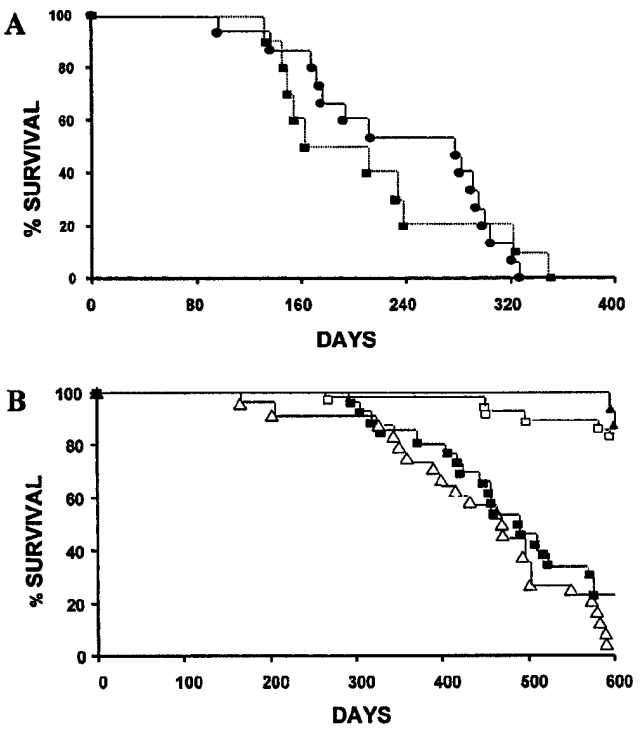

Fig. 6. Kaplan-Meier survival curves. *A*, survival of $APC^{+/min}$ mice with wild-type (■) or deleted (●) *CD44* gene. All $APC^{+/+}$ mice survived the 420-day period of observation. *B*, survival of $trp53^{+/tm1}$ mice. Within 600 days, mice with the $trp53^{+/tm1}$ genotype succumbed to various tumors independently of their CD44 status, leading to survival of 4% or 1 mouse ($CD44^{+/+}$, △) and 23% or 6 mice ($CD44^{-/-}$, ■). Control mice with the wild-type *trp53* gene had survival rates of 87% or 14 mice ($CD44^{+/+}$, ▲) and 83% or 30 mice ($CD44^{-/-}$, □).

CD44 gene products virtually abrogates osteosarcoma metastasis. In contrast, we did not find evidence for a role of CD44 in tumor growth or survival.

In various cancers, expression of CD44 splice variants is necessary and sufficient to cause metastasis formation (1, 2). In the present study, the role of CD44 in malignancy of nonhematopoietic origin was limited to inducing dissemination (Fig. 1) and corroborating the role of *CD44* as a metastasis gene in solid tumors. The expression of CD44 is sufficient to confer metastatic properties to primary osteosarcoma cells, but the expression of multiple CD44 variants on intestinal adenomatous polyps (15) is not associated with invasive growth. This may be accounted for by the dominance of metastasis suppressor gene products over gene products that induce dissemination (18). Intestinal cells frequently express the adhesion molecule DCC, which may prevent CD44-mediated invasion. Alternatively, metastasis formation by the intestinal polyps may be suppressed by elevated β-catenin, secondary to loss of APC protein function. This decreases enterocyte crypt villus migration (19) and may prevent invasive behavior. Osteosarcoma cells, in contrast, do not display any prominent expression of metastasis suppressor genes so that the expression of CD44 variants is sufficient to cause a malignant phenotype.

Various genetic influences can affect tumor multiplicity in $APC^{+/min}$ mice. They include genes for cell cycle control, DNA repair, and metalloproteinases. The genetic modifier Mom1 encodes a secretory phospholipase, Pla2g2a, expressed throughout the intestinal tract. The active allele of Pla2g2a leads to a reduction in the growth rate and multiplicity of intestinal adenomas (20). $APC^{+/min}$ mice homozygous for a null allele of p53 developed significantly more intestinal adenomas than those homozygous for the wild-type allele of p53. Similarly, the intact DNA mismatch repair gene *Pms2* reduces the number of intestinal tumors as compared with mice with a targeted deletion of this gene (21). In contrast, deletion of the gene for the metalloproteinase Matrilysin leads to substantial reduction in intestinal tumors, despite a lack of destruction of the basement membrane by these polyps (22). The intestinal polyps caused by the *APC* gene mutation express various splice variants of CD44 at the earliest stages of transformation, diagnosed as aberrant crypt foci with dysplasia (15); however, the contributions by *CD44* gene products to the pathogenesis of the intestinal polyps were unknown. In this study, the numbers of polyps and associated life spans were not influenced by the absence of *CD44* gene products. The size of the individual polyps did not appear to be compromised.

The expression of CD44 on tumors has been described to not only affect metastatic spread but also tumor growth (4, 5) and induction of oncogenes, such as *ras* (10). This opened the possibility that deletion of the *CD44* gene might influence disease progression. In $APC^{+/min}$ mice, the incidence of polyps and associated life spans were, however, not altered. Similarly, incidence, survival, and tumor weight of sarcomas in $trp53^{+/tm1}$ mice were not influenced by the absence of CD44, arguing against a prominent role for CD44 in early transformation or tumor growth. In contrast, the dissemination of osteosarcomas was virtually abrogated by the absence of *CD44* gene products (29 microscopically and macroscopically identified metastases in 6 $CD44^{+/+}$ mice, compared with 1 metastasis identified in 4 $CD44^{-/-}$ mice). We have found previously metastasis gene products to constitute a unique group of cancer-related biomolecules, which is distinct from growth controlling oncogene or tumor suppressor gene products. They are dysregulated in cancer at the levels of gene expression or mRNA splicing (18). The present results confirm the role of CD44 as a metastasis gene and refine our insights into the contributions of CD44 to cancer.

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REFERENCES

1. Gunthert, U., Hofmann, M., Rudy, W., Reber, S., Zoller, M., Haussmann, I., Matzku, S., Wenzel, A., Ponta, H., and Herrlich, P. A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. Cell, *65:* 13–24, 1991.
2. Seiter, R., Arch, R., Reber, S., Komitowski, D., Hofmann, M., Ponta, H., Herrlich, P., Matzku, S., and Zoller, M. Prevention of tumor metastasis formation by anti-variant CD44. J. Exp. Med., *177:* 443–455, 1993.
3. Weber, G. F., Ashkar, S., Glimcher, M. J., and Cantor, H. Receptor-ligand interaction between CD44 and osteopontin (Eta-1). Science (Wash. DC), *271:* 509–512, 1996.
4. Takahashi, K., Stamenkovic, I., Cutler, M., Saya, H., and Tanabe, K. K. CD44 hyaluronate binding influences growth kinetics and tumorigenicity of human colon carcinomas. Oncogene, *11:* 2223–2232, 1995.
5. Yu, Q., Toole, B. P., and Stamenkovic, I. Induction of apoptosis of metastatic mammary carcinoma cells *in vivo* by disruption of tumor cell surface CD44 function. J. Exp. Med., *186:* 1985–1996, 1997.
6. Fitzgerald, K. A., Bowie, A. G., Skeffington, B. S., and O'Neill, L. A. Ras, protein kinase C ζ, and I κ B kinases 1 and 2 are downstream effectors of CD44 during the activation of NF-κ B by hyaluronic acid fragments in T-24 carcinoma cells. J. Immunol., *164:* 2053–2063, 2000.
7. Choi, S. H., Takahashi, K., Eto, H., Yoon, S. S., and Tanabe, K. K. CD44s expression in human colon carcinomas influences growth of liver metastases. Int. J. Cancer, *85:* 523–526, 2000.
8. Lewin, D. I. New reports blur image of promising metastasis marker. J. NIH Res., *8:* 24–26, 1996.
9. Skelton, T. P., Zeng, C., Nocks, A., and Stamenkovic, I. Glycosylation provides both stimulatory and inhibitory effects on cell surface and soluble CD44 binding to hyaluronan. J. Cell Biol., *140:* 431–446, 1998.
10. Su, L. K., Kinzler, K. W., Vogelstein, B., Preisinger, A. C., Moser, A. R., Luongo, C., Gould, K. A., and Dove, W. F. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the *APC* gene. Science (Wash. DC), *256:* 668–670, 1992.
11. Schmits, R., Filmus, J., Gerwin, N., Senaldi, G., Kiefer, F., Kundig, T., Wakeham, A., Shahinian, A., Catzavelos, C., Rak, J., Furlonger, C., Zakarian, A., Simard, J. J., Ohashi, P. S., Paige, C. J., Gutierrez-Ramos, J. C., and Mak, T. W. CD44 regulates

hematopoietic progenitor distribution, granuloma formation, and tumorigenicity. Blood, *90:* 2217–2233, 1997.
12. Jacks, T., Remington, L., Williams, B. O., Schmitt, E. M., Halachmi, S., Bronson, R. T., and Weinberg, R. A. Tumor spectrum analysis in p53-mutant mice. Curr. Biol., *4:* 1–7, 1994.
13. Moen, C. J., Snoek, M., Hart, A. A., and Demant, P. *Scc-1*, a novel colon cancer susceptibility gene in the mouse: linkage to CD44 (Ly-24, Pgp-1) on chromosome 2. Oncogene, *7:* 563–566, 1992.
14. van Wezel, T., Stassen, A. P., Moen, C. J., Hart, A. A., van der Valk, M. A., and Demant, P. Gene interaction and single gene effects in colon tumour susceptibility in mice. Nat. Genet., *14:* 468–470, 1996.
15. Wielenga, V. J., Smits, R., Korinek, V., Smit, L., Kielman, M., Fodde, R., Clevers, H., and Pals, S. T. Expression of CD44 in Apc and Tcf mutant mice implies regulation by the WNT pathway. Am. J. Pathol., *154:* 515–523, 1999.
16. Taddesse-Heath, L., and Morse, H. C., Jr. Lymphomas in genetically engineered mice. *In:* J. M. Ward, J. F. Mahler, R. R. Maronpot, J. P. Sundberg, and R. M. Frederickson (eds.), Pathology of Genetically Engineered Mice, pp. 385–381. Ames, IA: Iowa State U. Press, 2000.
17. Fredrickson, T. N., and Harris, A. W. (eds.). Atlas of Mouse Hematopathology. Harwood Academic Publishers, Australia: , 2000.
18. Weber, G. F., and Ashkar, S. Stress response genes: the genes that make cancer metastasize. J. Mol. Med., *78:* 404–408, 2000.
19. Mahmoud, N. N., Boolbol, S. K., Bilinski, R. T., Martucci, C., Chadburn, A., and Bertagnolli, M. M. *Apc* gene mutation is associated with a dominant-negative effect upon intestinal cell migration. Cancer Res., *57:* 5045–5050, 1997.
20. Halberg, R. B., Katzung, D. S., Hoff, P. D., Moser, A. R., Cole, C. E., Lubet, R. A., Donehower, L. A., Jacoby, R. F., and Dove, W. F. Tumorigenesis in the multiple intestinal neoplasia mouse: redundancy of negative regulators and specificity of modifiers. Proc. Natl. Acad. Sci. USA, *97:* 3461–3466, 2000.
21. Baker, S. M., Harris, A. C., Tsao, J. L., Flath, T. J., Bronner, C. E., Gordon, M., Shibata, D., and Liskay, R. M. Enhanced intestinal adenomatous polyp formation in $Pms2^{-/-}$;Min mice. Cancer Res., *58:* 1087–1089, 1998.
22. Wilson, C. L., Heppner, K. J., Labosky, P. A., Hogan, B. L., and Matrisian, L. M. Intestinal tumorigenesis is suppressed in mice lacking the metalloproteinase matrilysin. Proc. Natl. Acad. Sci. USA, *94:* 1402–1407, 1997.

Phosphorylation-Dependent Interaction of Osteopontin with its Receptors Regulates Macrophage Migration and Activation

Georg F. Weber[2,3*#], Samer Zawaideh[1], Sherry Hikita[1], Vikram A. Kumar[2], Harvey Cantor[2,4], Samy Ashkar[1*]

[1]Laboratory for Skeletal Disorders and Rehabilitation, Department of Orthopedic Surgery, Children's Hospital, Harvard Medical School, 300 Longwood Avenue, Boston, MA 02115

[2]Department of Cancer Immunology & AIDS, Dana-Farber Cancer Institute, Departments of Medicine[3] and Pathology[4], Harvard Medical School, 44 Binney Street, Boston, MA 02115

* Corresponding authors.

Current address: Department of Radiation and Cancer Biology, New England Medical Center, NEMC #824, 750 Washington Street, Boston, MA 02111

ABSTRACT

Neutrophil-independent macrophage responses are a prominent part of delayed-type immune and healing processes and depend on T-cell secreted cytokines. An important mediator in this setting is the phosphoprotein osteopontin whose secretion by activated T-cells confers resistance to infection by several intracellular pathogens through recruitment and activation of macrophages. Here we analyze the structural basis of this activity following cleavage of the phosphoprotein by thrombin into two fragments. An interaction between the C-terminal domain of osteopontin and the receptor CD44 induces macrophage chemotaxis, while engagement of β_3-integrin receptors by a non-overlapping N-terminal osteopontin domain induces cell spreading and subsequent activation. Serine phosphorylation of the osteopontin molecule on specific sites is required for functional interaction with integrin but not CD44 receptors. Thus, in addition to regulation of intracellular enzymes and substrates, phosphorylation also regulates the biological activity of secreted cytokines. These data, taken as a whole, indicate that the activities of distinct osteopontin domains are required to coordinate macrophage migration and activation and may bear on incompletely understood mechanisms of delayed type hypersensitivity, wound healing, and granulomatous disease.

Short title: Osteopontin in macrophage activation

Key terms: Phosphorylation, integrins, delayed type hypersensitivity, CD44, metalloproteases

INTRODUCTION

Macrophages participate in two general types of inflammatory reactions. In immediate responses, macrophages are attracted and activated by cytokines secreted from neutrophils and support a reaction that usually is characterized by excessive fibrosis and scar formation. In contrast, delayed type hypersensitivity responses are largely neutrophil-independent, involve macrophage activation through other cytokines, and include inflammation associated with resistance to infection by intracellular pathogens and enhancement of wound healing. Differential expression of osteopontin by T-cells determines the relative levels of these two responses. Resistance to *Rickettsia* infection, mediated by vigorous induction of osteopontin, leads to an early monocyte influx into infected sites and rapid acquisition of macrophage bacteriocidal activity. Susceptibility to *Rickettsial* infection, on the other hand, reflects delayed and weak osteopontin responses and is characterized by an early accumulation of neutrophils at sites of infection [1,2]. High levels of osteopontin expression are also a hallmark of monocytic granulomatous reactions in the context of tuberculosis and silicosis [3]. In experimental glomerulonephritis, neutralizing antibodies to osteopontin greatly reduced the influx of macrophages and T-cells and reduced kidney damage [4]. Osteopontin may play a central role in bone healing [5,6], in stroke [7], and in the revascularization process that is essential for wound healing [8,9].

Osteopontin has been implicated in both cell attachment [10,11,12] and cell motility [13,14]. The protein binds to two types of receptors. Engagement of the homing receptor CD44 through a non-RGD cell binding domain of osteopontin is sufficient to induce chemotaxis or attachment [14,15]. Binding of osteopontin to $\alpha_v\beta_3$ integrin receptors via its Gly-Arg-Gly-Asp-Ser

(GRGDS) motif [12,16,17] may contribute to osteoclast adherence and resorption of bone [18] as well as to haptotaxis of endothelial cells [19], vascular smooth muscle cells [17,20], myelomonocytic cells [21], and tumor cells [22]. Vascular smooth muscle cells may also engage osteopontin through $\alpha_v\beta_1$ and $\alpha_v\beta_5$ integrins in an interaction that leads to adhesion of cells but not to migration [13], and adhesion to immobilized osteopontin via integrins α_4 and α_5 has been reported [21]. Engagement of integrin $\alpha_9\beta_1$ may induce migration [23]. Osteopontin is secreted in non-phosphorylated [24,25,26,27] and phosphorylated forms [28,29,30] that contain up to 28 phosphate residues and are differentially induced by tumor promoters and cytokines. Phosphorylation is functionally important because it may determine whether osteopontin associates with the cell surface or with the extracellular matrix [31,32]. Furthermore, cleavage of osteopontin with thrombin may enhance its cell attachment properties [33]. These results suggest that osteopontin may elicit specific cellular responses depending on its post-translational modification and the cell surface receptor repertoire on its target cell.

The structural basis for the interaction of osteopontin with macrophages leading to migration and perhaps activation is incompletely understood because earlier studies have often equated function with cell adherence. Prior experiments were performed mostly with cells of different lineages and it has been shown for other extracellular matrix proteins, including thrombospondin and fibronectin, that their interactions with macrophages could not be predicted from such studies. The necessity for cell attachment of the RGD motif in recombinant osteopontin has been confirmed in mutational analysis. Mutagenesis of the RGD sequence to RAA completely abrogated the interaction of melanoma cells with osteopontin while mutagenesis of the RGD to RGE resulted in 50% reduction in the attachment of these cells to osteopontin [23]. Mutagenizing

the RGD to RGE in mouse osteopontin eliminated the attachment of both tumor cells and gingival fibroblasts [34]. Analyses of further structural requirements for osteopontin functions have also demonstrated that phosphorylation may be essential for integrin-mediated cell adhesion [32] and may confer attachment of osteoclasts. It has not been clear whether additional sequences are necessary for osteopontin interaction with its integrin receptors and whether such sequences determine a second binding site or steric modifiers that expose the RGD sequence after phosphorylation. The role of osteopontin phosphorylation in other functions has not been investigated. We have recently found phosphorylation to be essential for various cytokine functions of osteopontin [35,36,37]. Here we show that engagement of β_3-integrin and CD44 receptors by separate domains of osteopontin leads to the expression of distinct macrophage response phenotypes which can be separated on the level of biological function and that the interaction of integrin receptors with osteopontin is regulated by phosphorylation of specific sites on the ligand. We examine macrophages as the cell type that is physiologically predominantly affected by osteopontin and we compare native osteopontin to recombinant protein that has been phosphorylated on specific sites.

MATERIALS AND METHODS

Cell lines. MH-S is a murine macrophage cell line that was derived by SV40 transformation from an adherent cell enriched population of alveolar macrophages (CRL-2019, ATCC). MT-2/1 is a thymus-derived macrophage from a Balb/c mouse that was immortalized by infection with retroviral vector [38]. In both cell lines, expression of CD44 and integrin β_3 were measured by flow cytometry with FITC-labeled antibodies IM7 and 2C9.G2. CD44 variant expression was analyzed by RT-PCR with previously published primer sets [39]. A31 is an integrin $\alpha_v\beta_3^-$, CD44$^-$ murine embryonic fibroblast clone derived from Balb/c 3T3 cells (CCL-163, ATCC). A31 cells transfected with CD44 (A31.C1) or A31 mock transfectants were generated as described [14].

Osteopontin purification and cleavage. Recombinant mouse GST-osteopontin fusion protein was derived from E. coli, digested with factor Xa and purified by affinity chromatography, as described [40,41]. Osteopontin was purified to homogeneity as determined by N-terminal sequencing. The native osteopontin used in this study was isolated from bone cell cultures or from MC3T3E1 cells and is a full-length protein that is O-glycosylated and highly sialylated, free of sulfate or N-glycosylation, and contains 15-17 phosphate residues. Thrombin cleavage and phosphorylation of either the dephosphorylated native protein or recombinant osteopontin was accomplished by human thrombin (Sigma Chemicals), protein kinase A, protein kinase G, Golgi kinases or purified casein kinase II or casein kinase I as described [12,29,30,40,41,42]. Dephosphorylation was performed using type II potato acid phosphatase [35]. Endotoxin levels of purified osteopontin were below 1 ng/g according to Limulus lysate analysis [35].

In preliminary experiments, MH-S cells attached to, but did not spread on phosphorylated and unphosphorylated PNGRGDSLA*Y*GLR synthetic peptides. We therefore attempted to isolate a

proteolytic fragment that can promote cell spreading. Neither partial tryptic, chymotryptic or Asp-N endopeptidase digestion of osteopontin resulted in the isolation of an active peptide, however, a 10 kD fragment was isolated from a Lys-C digest that mediated the spreading of macrophages at approximately 40 % (mol/mol) the activity of the N-terminal thrombin fragment. The N-terminal sequence of this peptide was determined to be QETLPSN. Based on size and sequence analysis of this peptide, referred to as NT10k, we predict that it terminates at the thrombin cleavage site. It contains seven potential phosphorylation sites and approximately 5 mols of phosphate/mol of peptide. Upon dephosphorylation of this peptide, spreading activity is lost but can be regained by rephosphorylation with Golgi kinases.

Chemotaxis. Directed migration of cells was determined in multi-well chemotaxis chambers [14,43]. Two-well culture plates (Transwell) with polycarbonate filters (pore size 8-12 μm) separating top and bottom wells were coated with 5 μg fibronectin. 2 X 10^5 cells were added to the upper chamber and incubated at 37° C in the presence or absence of osteopontin in the lower chamber. After 4 h, the filters were removed, fixed in methanol, stained with hematoxylin and eosin and cells that had migrated to various areas of the lower surface were counted microscopically. Controls for chemokinesis included 200 ng of the appropriate form of osteopontin in the top well. For inhibition studies, the cells were incubated with the relevant antibodies for 15 min before adding to the upper well of the transwell chamber. All assays were done in triplicates and are reported as mean ± standard error.

Haptotaxis. Haptotaxis of monocytic cell lines to osteopontin or fragments of osteopontin was assayed using a Boyden chamber. The lower surface or both sides of polycarbonate filters with 8 μm pore size were coated with the indicated amounts of osteopontin. 2 X 10^5 cells were added to the upper chamber, and incubated at 37° C in the absence of any factors in the lower chamber. After

4 h, the filters were removed, fixed in methanol and stained with hematoxylin and eosin. Cells that had migrated to the lower surface were counted under a microscope. All assays were done in triplicates and are reported as mean ± standard error.

***In vivo* cell migration.** Female C57BL/6 mice, purchased from Jackson Laboratories and housed at the Redstone Animal Facility of the Dana-Farber Cancer Institute, were injected intraperitoneally with 200 µl of PBS containing varying dosages of K7 osteosarcoma-derived osteopontin. Injections of vehicle alone (PBS) served as negative controls. Mice were sacrificed by CO_2-asphyxiation at varying times after injection followed by immediate collection of peritoneal exsudate by intraperitoneal injection and recovery of twice 10 mL PBS. Red blood cells were removed by hypotonic lysis with ACK buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4) for 5 minutes at room temperature. Cells were washed and resuspended in DMEM containing 5% FBS for fluorescent antibody staining at a concentration of 0.2 to 1 million cells in 50 µl. Fluorescence-labeled antibodies (1 µg/1x10^6 cells) were incubated with cells for 30 minutes at 4°C, before washing twice with 200 µl of PBS and fixation in 500 µl of 2% paraformaldehyde in PBS. Analysis for cellular expression of CD44 (Pgp-1, PE) together with CD11b (Mac-1, FITC, macrophage marker), B220 (FITC, B-cell marker), or CD3 (FITC, T-cell marker) was performed by dual-color flow cytometry with antibodies from PharMingen using a Coulter EPICS flow cytometer. Appropriate non-specific antibody controls and single color controls were included.

Cell attachment and spreading. Cell adhesion is a prerequisite for chemotaxis, haptotaxis and cell spreading. In vitro assays revealed that cells display both passive and active adhesion. Active adhesion is pH- and temperature-dependent, reduced by trypsin treatment and dependent on cell viability. Passive adherence is temperature- and pH-independent, unaffected by trypsin treatment

and independent of cell viability. Maximal levels of active adherence by macrophages depend on harvest of cells without enzymes from subconfluent cultures and limited exposure to temperature fluctuation. In these studies we distinguish among cell passive adhesion, active adhesion and spreading. Passive adhesion is not associated with rearrangement of the cytoskeleton: attached (possibly adhered) cells cannot undergo G0-G1 transition (as judged by cyclin D expression) and become non-viable within 6-12 hours. Actively adherent cells rearrange their cytoskeleton, can undergo G0-G1 transition and proliferate. Spread cells are arrested cells in G2, do not proliferate, and are characterized by focal adhesion plaques. Most dye binding assays cannot differentiate these different types of attachment. Dye binding assays, such as crystal violet, that bind very tightly to dead cells do not allow differentiation between cell debris and live, actively-attached cells.

24-well plates were coated overnight at 4°C with 10 μg/ml of the indicated ligand then blocked for 1 h at room temperature with 10 mg/ml BSA in PBS. At these concentrations, the osteopontin-derived ligands are several orders of magnitude in excess of the estimated numbers of receptors on the plated cells and are considered saturating, so that moderate differences in ligand binding to the plastic do not affect the experiment. To preserve the integrity of adhesion receptors MH-S monocytic cells were harvested from subconfluent cultures by non-enzymatic cell dissociation solution (Sigma, St Louis MO). Cells were washed twice with PBS and resuspended at a concentration of 1 X 10^5 cells/ml of sterile Ca^{2+}- and Mg^{2+}-free PBS supplemented with 0.1% BSA and 1 mM sodium pyruvate. 5 x 10^4 cells were incubated in each well and, after 1 h at 37°C, the wells were washed 3 times with 0.5 ml PBS to remove non-adherent cells, fixed in 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 1 hour, then stained with toluidine blue and hematoxylin. The total number of attached or spread cells in each well were counted microscopically using a Nikon Eclipse microscope equipped with a Sony digital Camera.

Total number of attached or spread cells were quantitated using Optima 5.2 image analysis system. Each experiment was done in triplicates and is reported as mean ± standard error. To minimize variability inherent to cell attachment studies we scored cells as attached only when a defined nucleus was observed accompanied by a transition from round to cuboidal cell morphology. Round cells are loosely attached with no defined nucleus and were scored as non-attached. These cells can be removed with repeated washes. The viability of the cells was measured before and after the termination of the experiments and only data from experiments with greater than 95% cell viability were used. Further, under the conditions used in these experiments, cell attachment was temperature-dependent, inhibitable by trypsin treatment and not affected by inhibitors of protein synthesis or secretion. Cell spreading was determined by membrane contour analysis and was scored according to increase in cell volume/surface area. In some experiments, cell spreading was also assessed by the formation of stress fibers. Each experiment was performed in quadruplicate wells and repeated 3 times.

Zymography Secretion of proteinases was assayed by SDS-substrate gel electrophoresis under non-reducing conditions as described [44,45]. Cell culture supernatant was collected after 6 hours of culture, concentrated 5 times and resuspended in 200 μl zymogram buffer (40 mM Tris, pH 7.5) before addition to Laemmli sample buffer and electrophoresis in 10% polyacrylamide gels impregnated with 1 mg/ml gelatin. Following electrophoresis, gels were incubated for 30 min at 37°C in 50 ml of 50 mM Tris-HCl buffer, pH 8.0, containing 2% Triton-X100 and 10 mM $CaCl_2$ to remove the SDS, followed by incubation for 18 h in 50 mM Tris-HCl buffer, pH 8.0, containing 5 mM $CaCl_2$. After staining the gels with Coomassie Brilliant Blue, gelatin degrading enzymes were identified as clear bands against a dark blue background.

RESULTS

Osteopontin interacts with $CD44^+$ and $CD44^-$ macrophages *in vivo*

Singh et al. [16] have reported an RGD-dependent interaction between osteopontin and integrin receptors and *in vivo* attraction of macrophages by subcutaneously injected osteopontin. We analyzed osteopontin-attracted macrophage populations for expression of CD44. Titration of soluble osteopontin into the peritoneum resulted in a dose-dependent increase in the cellular infiltrate (**Figure 1A,B**). According to our preliminary experiments, the peak response occurred at 4-6 hrs at about 10 μg of osteopontin; the decreased response after 6 hours may reflect clearance of soluble osteopontin from the peritoneal cavity. The numbers of Mac-1^+ cells in the infiltrate were increased five-fold over basal levels and about 90% of peritoneal macrophages were $CD44^+$. Osteopontin injections induced a disproportionately high increase in both Mac-1^+CD44^+ (5.27 ± 1.14 fold at 10 μg osteopontin) as well as Mac-1^+CD44^- cells (6.27 ± 2.28 fold), implying that ligation of both classes of osteopontin receptors may contribute to migration *in vivo*. The predominant migration of Mac-1^+ cells (about 65% of the infiltrate) is consistent with previous studies of osteopontin [16], and is in contrast to the inflammatory response to lipopolysaccharide, which consists of roughly equal numbers of $CD3^+$, $B220^+$, and Mac-1^+ cells (data not shown). This cellular infiltration is unlikely to reflect contamination of osteopontin, which was pure according to peptide sequence analysis, since co-injection of osteopontin with anti-osteopontin antibody prevented the influx of cells whereas rabbit immunoglobulin had no effect (data not shown).

Our previous investigations of stable transfectants of A31 cells indicated that the interaction of osteopontin with CD44 depended on expression of CD44 splice variants 3-6 [14], which characterize activated lymphocytes [46]. More recent studies indicate that A31 cells transfected

with the standard form of CD44 (lacking variant exons) do not bind osteopontin (unpublished results and [47]). These findings are consistent with the observation that lymphocytes attracted into the peritoneal cavity appear to be activated as judged by increased forward scatter measurements in flow cytometry.

The C-terminal domain of osteopontin interacts with CD44 to induce chemotaxis

Earlier studies of osteopontin binding to the myelomonocytic cell line Wehi 3 suggested that this interaction depended mainly on engagement of integrin receptors [16] while later investigations identified CD44 as a second receptor that was associated with chemotaxis [14]. Since thrombin cleaves osteopontin in two sites releasing two large fragments, an N-terminal 1-153 fragment containing the RGD motif and a 158-294 C-terminal fragment [33] and since previous studies have demonstrated that the attachment of tumor cells is mediated preferentially by RGD-containing domains of osteopontin [48], we exploited these observations to determine whether distinct domains of osteopontin were responsible for engagement of the two receptors. The macrophage cell lines MT-2/1 and MH-S express the relevant receptors integrin β_3 and CD44 at high levels according to flow cytometry. Although one form containing variant exons appears to be the most prominent CD44 gene product as judged by PCR with primers for the constitutive exons 5 and 16, three bands between 200 and 400 base pairs are amplified with primers for exon 5 and exon v6. Expression of standard CD44 was not detected **(Figure 2)**.

The CD44 transfected cell line A31.C1 migrated toward soluble osteopontin or the C-terminal fragment, but not toward the N-terminal fragment **(Table 1A)**. The vector transfected control does not chemotax toward osteopontin [14] confirming that the C-terminal domain of osteopontin interacts with CD44. The C-terminal fragment of osteopontin also induced chemotaxis

of the macrophage cell line MH-S as efficiently as intact osteopontin, while the N-terminal 30 kDa osteopontin fragment was inactive (**Table 1B-E**); equimolar mixtures of both fragments displayed activity similar to that of the 28 kDa C-terminal fragment alone (data not shown). Chemotaxis induced by osteopontin or its C-terminal domain was inhibitable by anti-CD44 antibody, but not by GRGDS peptide or anti-integrin β_3 antibody. These results, taken together, indicate that 28 kDa C-terminal part of the molecule is sufficient to induce CD44-dependent chemotaxis.

The N-terminal domain of osteopontin induces haptotaxis of macrophages via integrin β_3

Cells can move up a gradient of immobilized ligand, this cell crawling may occur on vessel walls or in the interstitium and is referred to as haptotaxis. We assessed the contribution of interactions between immobilized osteopontin and integrin receptors to cell motility. The ability of the immobilized ligand to induce monocyte haptotaxis was judged by cell migration through polycarbonate filters. Osteopontin induced monocyte migration that was mainly directional (i.e., the cells responded to a positive gradient of bound osteopontin) (**Table 1C**), and thus haptotactic, and was inhibited by GRGDS and antibody to the β_3 chain of integrins but not by antibody to CD44 (**Table 1D**).

Since some osteopontin functions are dependent on the phosphorylation of the ligand, we attempted to localize the critical residues for haptotaxis. Phosphorylation has to occur at specific sites because Golgi kinases and casein kinases I or II can activate osteopontin whereas protein kinases A or G phosphorylate the recombinant molecule but do not confer integrin binding (**Table 1E**). Earlier studies which showed that RGD-containing peptides can confer function may have induced non-specific effects through multiple integrin receptors. Our data demonstrate that the

RGD motif is necessary but not sufficient to confer specific osteopontin function, a sequence N-terminal to the RGD sequence is also needed.

The N-terminal domain of osteopontin induces spreading and activation of macrophages via integrin β_3

Macrophage spreading on extracellular matrix proteins depends, in part, on engagement of their integrin receptors. Spreading of the MH-S macrophage cell line on immobilized native osteopontin is mediated by the RGD-containing N-terminal thrombin cleavage fragment but not by the C-terminal fragment and is reversed by addition of soluble GRGDS but not control GRGES peptide. Moreover, phosphorylation of recombinant osteopontin is required for this activity **(Figure 3).**

Osteopontin induces pro-inflammatory mediators through integrin β_3

Macrophage spreading is often associated with cellular activation. Here we analyze the effects of osteopontin on macrophage production of effector cytokines including several pro-inflammatory mediators and metalloproteinases. Metalloproteinases, which regulate cell motility and invasion, represent a family of more than 14 proteolytic enzymes that have distinct but overlapping substrate specificity. Metalloprotease 2 and 9 cleave type IV collagen and are thought to be essential for efficient migration of monocytes through basement membranes. The phosphorylated N-terminal fragment but not the C-terminal fragment of osteopontin induced strong MMP-9 (gelatinase B) responses **(Figure 4)** and induction was inhibited by GRGDS but not GRGES (data not shown). Consistent with its role in delayed type hypersensitivity responses, osteopontin induced IL-12 and TNF-α, but not IL-1β, IL-10 or IL-6. Additional analysis revealed

that cytokine induction depends on the interaction between phosphorylated osteopontin and peritoneal macrophages (**Figure 5**). This interaction was mediated by the N-terminal portion of osteopontin and was inhibited by GRGDS and integrin β_3 antibody (not shown).

DISCUSSION

The present structure-function analysis of osteopontin demonstrates that engagement of its two main receptors by distinct domains leads to differential monocyte responses. Chemoattractant activity resides within the C-terminal region of osteopontin and is mediated by CD44. In contrast, the RGD motif within a non-overlapping N-terminal portion of osteopontin, exposed after phosphorylation or thrombin cleavage, can induce haptotaxis and cellular activation. Thus, in addition to the well-established role of phosphorylation in regulating the biological interactions of intracellular enzymes and their substrates, phosphorylation of a secreted extracellular protein also can provide molecular information that targets it to particular interaction partners, which dictate its biological activity. In vivo, additional components may contribute to regulating osteopontin biology. Several other integrin receptors are engaged by osteopontin [13,21]. Furthermore, CD44 may be ligated individually or in association with integrin β_1 [47]. This interaction is not likely to contribute to chemotaxis because the integrin β_1 binding sites on osteopontin have been mapped outside the C-terminal fragment used in our experiments, however, other in vivo functions by co-ligated CD44 and integrin β_1 are possible. The combined contributions by all receptors may underlie the profound effects of osteopontin on cellular immunity.

The participation of monocytes/macrophages in inflammation entails emigration of these cells from peripheral blood into infected tissues, where they produce cytokines that regulate diverse processes including anti-microbial activity, cell growth, differentiation and wound healing [49]. Emigration of monocytes depends on coordinate engagement of different subsets of cell surface receptors by cytokines and extracellular matrix that underlie migration or adhesion within various anatomic compartments. Osteopontin plays an important role in macrophage infiltration in response

to pathological stimuli [50]. Our findings suggest that attraction and activation of monocytes depends, in part, on orchestrated activities of osteopontin binding domains that have been modified by thrombin [22], ecto-kinases [51], and ecto-phosphatases [32]. Thrombin cleavage of osteopontin that has been integrated into the matrix through transglutaminases [52] can lead to the release of chemotactic C-terminal osteopontin fragment and attraction of monocytes to a site of infection. Subsequent engagement of integrin receptors on emigrant monocytes by immobilized phosphorylated N-terminal domain of osteopontin may facilitate local haptotactic migration towards a site of microbial infection or inflammation (**Figure 6**). After arrival, attachment and spreading of emigrant monocytes mediated by engagement of $\alpha_v\beta_3$ integrin receptors by the N-terminal osteopontin thrombin cleavage product (possibly through increased access of the RGD binding site [19]), can lead to macrophage activation and expression of inflammatory mediators such as metalloproteases and cytokines (**Figure 5**). The cytokine profile reflects a type 1 pattern and, in conjunction with the coordinated regulation of macrophage migration and invasion, accounts for the prominent role played by the cytokine osteopontin in cellular immunity [35]. The coupling of macrophage cell shape and gene expression through the linkage of cytoskeletal networks to the extracellular matrix provides a molecular framework for differential responses to various presentations of the same ligand [53]. Although additional *in vivo* studies are required to test this model, the definition of the functional domains of osteopontin in this report represents an important step in understanding this process and may allow the rational development of osteopontin analogs that antagonize or mimic discrete biological activities of the parent molecule.

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REFERENCES

[1] Patarca, R., Freeman, G.J., Singh, R.P., Wei, F.-Y., Durfee, T., Blattner, F., Regnier, D.C., Kozack, C.A., Mock, B.A., Morse, H.C. III, Jerrells, T.R., Cantor, H. (1989) Structural and functional studies of the early T lymphocyte activation 1 (Eta-1) gene. Definition of a novel T cell-dependent response associated with genetic resistance to bacterial infection. J. Exp. Med. **170**, 145-161.

[2] Patarca, R., Singh, R.P., Wei, F.-Y., Iregui, M.V., Singh, P., Schwartz, J., Cantor, H. (1990) Alternative pathways of T-cell activation and positive clonal selection. Immunol. Rev. **116**, 1-16.

[3] Nau, G.J., Guilfoile, P., Chupp, G.L., Berman, J.S., Kim, S.J., Kornfeld, H., Young, R.A. (1997) A chemoattractant cytokine associated with granulomas in tuberculosis and silicosis. Proc. Natl. Acad. Sci. USA **94**, 6414-6419.

[4] Yu, X.Q., Nikolic-Paterson, D.J., Mu, W., Giachelli, C.M., Atkins, R.C., Johnson, R.J., Lan, H.Y. (1998) A functional role for osteopontin in experimental crescentic glomerulonephritis in the rat. Proc. Am. Assoc. Phys. **110**, 50-64.

[5] McKee, M.D., Nanci, A. (1996) Osteopontin: an interfacial extracellular matrix protein in mineralized tissues. Connect Tissue Res. **35**, 197-205.

[6] Nakase, T., Sugimoto, M., Sato, M., Kaneko, M., Tomita, T., Sugamoto, K., Nomura, S., Kitamura, Y., Yoshikawa, H., Yasui, N., Yonenobu, K., Ochi, T. (1998) Switch of osteonectin

and osteopontin mRNA expression in the process of cartilage-to-bone transition during fracture repair. Acta Histochem. **100**, 287-295.

[7] Wang, X., Louden, C., Yue, T.L., Ellison, J.A., Barone, F.C., Solleveld, H.A., Feuerstein, GZ. (1998) Delayed expression of osteopontin after focal stroke in the rat. J. Neurosci. **18**, 2075-2083.

[8] Liaw, L., Birk, D.E., Ballas, C.B., Whitsitt, J.S., Davidson, J.M., Hogan, B.L. (1998) Altered wound healing in mice lacking a functional osteopontin gene (spp1). J. Clin. Invest. **101**, 1468-1478.

[9] Giachelli, C.M., Liaw, L., Murry, C.E., Schwartz, S.M., Almeida, M. (1995) Osteopontin expression in cardiovascular diseases. Ann. NY Acad. Sci. **760**, 109-126.

[10] Oldberg, A., Franzen, A., Heinegard, D. (1986) Cloning and sequence analysis of rat bone sialoprotein (osteopontin) cDNA reveals an Arg-Gly-Asp cell-binding sequence. Proc. Natl. Acad. Sci. USA **83**, 8819-8823.

[11] Oldberg, A., Franzen, A., Heinegard, D., Pierschbacher, M.D., Ruohslahti, E. (1986) Identification of a bone sialoprotein receptor in osteosarcoma cells. J. Biol. Chem. **263**:19433-19436.

[12] Salih E, Ashkar S, Gerstenfeld LC, Glimcher MJ. (1996) Protein kinases of cultured

osteoblasts: selectivity for the extracellular matrix proteins of bone and their catalytic competence for osteopontin. J. Bone Miner. Res. **11**:1461-1473.

[13] Liaw, L., Skinner, M.P., Raines, E.W., Ross, R., Cheresh, D.A., Schwartz, S.M., Giachelli, C.M. (1995) The adhesive and migratory effects of osteopontin are mediated via distinct cell surface integrins. Role of alpha v beta 3 in smooth muscle cell migration to osteopontin in vitro. J. Clin. Invest. **95**, 713-724.

[14] Weber, G.F., Ashkar, S., Glimcher, M.J., Cantor, H. (1996) Receptor-ligand interaction between CD44 and Osteopontin/Eta-1. Science **271**, 509-512.

[15] van Dijk S., D'Errico, J., Somerman, M.J., Farach-Carson, M.C., Butler, W.T. (1993) Evidence that a non-RGD domain in rat osteopontin is involved in cell attachment. J. Bone Miner. Res. **8**, 1499-1506.

[16] Singh, R.P., Patarca, R., Schwartz, J., Singh, P., Cantor, H. (1990) Definition of a specific interaction between Eta-1 protein and murine macrophages in vitro and in vivo. J. Exp. Med. **171**, 1931-1942.

[17] Liaw, L., Almeida, M., Hart, C.E., Schwartz, S.M., Giachelli, C.M. (1994) Osteopontin promotes vascular cell adhesion and spreading and is chemotactic for smooth muscle cells in vitro. Circul. Res. **74**, 214-224.

[18] Denhardt, D.T., Guo, X. (1993) Osteopontin: a protein with diverse functions. FASEB J. **7**, 1475-1482.

[19] Senger, D.R., Ledbetter, S.R., Claffey, K.P., Papadopoulos-Sergiou, A., Peruzzi, C.A., Detmar, M. (1996) Stimulation of endothelial cell migration by vascular permeability factor/vascular endothelial growth factor through cooperative mechanisms involving the β3 integrin, osteopontin, and thrombin. Am. J. Pathol. **149**, 293-305.

[20] Yue, T.L., McKenna, P.J., Ohlstein, E.H., Farach-Carson, M.C., Butler, W.T., Johanson, K., McDevitt, P., Feuerstein, G.Z., Stadel, J.M. (1994) Osteopontin-stimulated vascular smooth muscle cell migration is mediated by beta 3 integrin. Exp. Cell Res. **214**, 459-464.

[21] Nasu, K., Ishida, T., Setoguchi, M., Higuchi, Y., Akizuki, S., Yamamoto, S. (1995) Expression of wild type and mutated rabbit osteopontin in Escherichia coli, and their effects on adhesion and migration of P388D1 cells. Biochem. J. **307**, 257-265.

[22] Senger, D.R., Perruzzi, C.A. (1996) Cell migration promoted by a potent GRGDS-containing thrombin cleavage fragment of osteopontin. Biochim. Biophys. Acta **1314**, 13-24.

[23] Smith, L.L., Giachelli, C.M. (1998) Structural requirements for alpha 9 beta 1-mediated adhesion and migration to thrombin-cleaved osteopontin. Exp. Cell Res. **242**, 351-360.

[24] Kubota, T., Zhang, Q., Wrana, J.L., Ber, R., Aubin, J.E., Butler, W.T., Sodek, J. (1989)

Multiple forms of SppI (secreted phosphoprotein; osteopontin) synthesized by normal and transformed rat bone cell populations: regulation by TGF-α. Biochem. Biophys. Res. Comm. **162**, 1453-1459.

[25] Chambers, A.F., Behrend, E.I., Wilson, S.M., Denhardt, D.T. (1992) Induction of expression of osteopontin in metastatic, ras-transformed NIH 3T3 cells. Anticancer Res. **12**, 43-47.

[26] Barak-Shalom, T., Schickler, M., Knopov, V., Shapira, R., Hurwitz, S., Pines, M. (1995) Synthesis and phosphorylation of osteopontin by avian epiphyseal growth-plate chondrocytes as affected by differentiation. Comp. Biochem. Physiol. **111**, 49-59.

[27] Chang, P.L., Prince, C.W. (1993) 1 alpha, 25-Dihydroxyvitamin D3 enhances 12-0-tetradecanoylphorbol-13-acetate-induced tumorigenic transformation and osteopontin expression in mouse JB6 epidermal cells. Cancer Res. **53**, 2217-2220.

[28] Sorensen, E.S., Petersen, T.E. (1994) Identification of two phosphorylation motifs in bovine osteopontin. Biochem. Biophys. Res. Comm. **198**, 200-205.

[29] Salih, E., Ashkar, S., Gerstenfeld, L.C., Glimcher, M.J. (1995) Identification of the in vivo phosphorylated sites of secreted osteopontin from chicken osteoblasts. Ann. NY Acad. Sci. **760**, 357-360.

[30] Salih, E., Ashkar, S., Gerstenfeld, L.C., Glimcher, M.J. (1997) Identification of the phosphorylated sites of metabolically ^{32}P-labelled osteopontin from cultured chicken osteoblasts. J. Biol. Chem. **272**, 13966-13973.

[31] Nemir, M., DeVouge, M.W., Mukherjee, B.B. (1989) Normal rat kidney cells secrete both phosphorylated and nonphosphorylated forms of osteopontin showing different physiological properties. J. Biol. Chem. **264**, 18202-18208.

[32] Ek-Rylander, B., Flores, M., Wendel, M., Heinegard, D., Andersson, G. (1994) Dephosphorylation of osteopontin and bone sialoprotein by osteoclastic tartrate-resistant acid phosphatase. Modulation of osteoclast adhesion in vitro. J. Biol. Chem. **269**, 14853-14856.

[33] Senger, D.R., Brown, L.F., Peruzzi, C.A., Papdopoulos-Sergious, A., van der Water, L. (1995) Osteopontin at the tumor/host interface. Functional regulation by thrombin cleavage and consequences for cell adhesion. Ann. NY Acad. Sci. **760**, 83-100.

[34] Xuan, J.W., Hota, C., Shigeyama, Y., D'Errico, J.A., Somerman, M.J., Chambers, A.F. (1995) Site-directed mutagenesis of the arginine-glycine-aspartic acid sequence in osteopontin destroys cell adhesion and migration functions. J. Cell Biochem. **57**, 680-690.

[35] Ashkar, S., Weber, G.F., Panoutsakopoulou, V., Sanchirico, M.E., Janssen, M., Zawaideh, S., Rittling, S., Denhardt, D.T., Glimcher, M.J., Cantor, H. (2000) The cytokine Eta-1/Opn is an essential molecular bridge to type 1 (cell-mediated) immunity. Science **287**, 860-864.

[36] Adler, B., Ashkar, S., Cantor, H., Weber, G.F. (2001) Costimulation by extracellular matrix proteins determines the response to TCR ligation. Cell. Immunol. **210**, 30-40.

[37] Weber, G.F., Adler, B., Ashkar, S. (1999) Osteopontin in oxidative stress responses. In: Ruffolo, R.R. Jr., Feuerstein, G.Z., Hunter, A.J., Poste, G., Metcalf, B.W. (Eds). Inflammatory Cells and Mediators in CNS Diseases. Amsterdam (Harwood Academic Publishers), 97-112.

[38] Lutz, M.B., Granucci, F., Winzler, C., Marconi, G., Paglia, P., Foti, M., Asmann, C.U., Cairns, L., Rescigno, M., Ricciardi-Castagnoli, P. (1994) Retroviral immortalization of phagocytic and dendritic cell clones as a tool to investigate functional heterogeneity. J. Immunol. Meth. **174**, 269-279.

[39] Stickeler, E., Kittrell, F., Medina, D., Berget, S.M. (1999) Stage-specific changes in SR splicing factors and alternative splicing in mammary tumorigenesis. Oncogene **18**, 3574-3582.

[40] Ashkar, S., Glimcher, M.J., Saavedra, R.A. (1993) Mouse osteopontin expressed in E. coli autophosphorylates tyrosine residues. Biochem. Biophys. Res. Comm. **194**, 274-279.

[41] Ashkar, S., Teplow, D.B., Glimcher, M.J., Saavedra, R.A. (1993) In vitro phosphorylation of mouse osteopontin expressed in E. coli. Biochem. Biophys. Res. Comm. **191**, 126-133.

[42] Ashkar, S., Schaffer, J.L., Salih, E., Gerstenfeld, L.C., Glimcher, M.J. (1995)

Phosphorylation of osteopontin by Golgi kinases. Ann. NY Acad. Sci. **760**, 296-298.

[43] Moses, M.A., Sudhalter, J., Langer, R. (1990) Identification of an inhibitor of neovascularization from cartilage . Science **248**, 1408-1410.

[44] Giunciuglio, D., Cai, T., Filanti, C., Manduca, P., Albini, A. (1995) Effect of osteoblast supernatants on cancer cell migration and invasion. Cancer Lett. **97**, 69-74.

[45] Unemori, E.N., Werb, Z. (1986) Reorganization of polymerized actin: a possible trigger for induction of procollagenase in fibroblasts cultured in and on collagen gels. J. Cell Biol. **103**, 21-1031.

[46] Arch, R, Wirth, K., Hofmann, M., Ponta, H., Matzku, S., Herrlich, P., Zoeller, M. (1992) Participation in normal immune responses of a metastatic inducing splice variant of CD44. Science **257**, 682-685.

[47] Katagiri, Y.U., Sleeman, J., Fujii, H., Herrlich, P., Hotta, H., Tanaka, K., Chikuma, S., Yagita, H., Okumura, K., Murakami, M., Saiki, I., Chambers, A.F., Uede, T. (1999) CD44 variants but not CD44s cooperate with beta1-containing integrins to permit cells to bind to osteopontin independently of arginine-glycine-aspartic acid, thereby stimulating cell motility and chemotaxis. Cancer Res **59**, 219-226.

[48] Xuan, J.W., Hota, C., Chambers, A.F. (1994) Recombinant GST-human osteopontin fusion

protein is functional in RGD-dependent cell adhesion. J. Cell. Biochem. **54**, 247-55.

[49] Singer, I.I., Kawka, D.W., Bayne, E.K., Donatelli, S.A., Weidner, J.R., Williams, H.R., Ayala, J.M., Mumford, R.A., Lark, M.W., Glant, T.T., et al. (1995) VDIPEN, a metalloproteinase-generated neoepitope, is induced and immunolocalized in articular cartilage during inflammatory arthritis. J. Clin. Invest. **95**, 2178-2186.

[50] Giachelli, C.M., Lombardi, D., Johnson, R.J., Murry, C.E., Almeida, M. (1998) Evidence for a role of osteopontin in macrophage infiltration in response to pathological stimuli in vivo. Am. J. Pathol. **152**, 353-358.

[51] Zhu, X.L., Luo, C., Ferrier, J.M., Sodek, J. (1997) Evidence of ectokinase-mediated phosphorylation of osteopontin and bone sialoprotein by osteoblasts during bone formation in vitro. Biochem. J. **323**, 637-643.

[52] Mukherjee, B.D., Nemir, M., Beninati, S., Cordella-Miele, E., Singh, K., Chackalaparampil, I., Shanmugam, V., DeVouge, M.W., Mukherjee, A.B. (1995) Interaction of osteopontin with fibronectin and other extracellular matrix molecules. Ann. NY Acad. Sci. **760**, 201-212.

[53] Singhvi, R., Kuman, A., Lopez, G.P., Stephanopoulos, G.N., Wang, D.L., Whitesides, G.M., Ingber, D.E. (1994) Engineered cell shape and function. Science **264**, 696-698.

LEGENDS TO TABLES & FIGURES

Table 1. Migratory activity of osteopontin and its thrombin fragments. A) Chemotaxis of A31.C1 cells, stably transfected with CD44. Soluble osteopontin or its cleavage fragments were added to the lower well of a Boyden chamber in the presence or absence of equal amounts of osteopontin or its fragments in the upper well. Values are expressed as total numbers of transmigrated cells (mean $\pm$ standard error), numbers in bold are significantly different from control values with $p < 0.01$ or better. Numbers in italics are significantly different from the value in the top row of the column (reflecting inhibition of maximally induced migration) with $p < 0.05$ or better. B) Chemotaxis of MH-S cells. Soluble osteopontin or its cleavage fragments were added to the Boyden chamber as described in A). Values are expressed as total numbers of transmigrated cells (mean $\pm$ standard error), numbers in bold are significantly different from control values with $p < 0.01$ or better. Numbers in italics are significantly different from the value in the top row of the column (reflecting inhibition of maximally induced migration) with $p < 0.05$ or better. C) Haptotactic response of MH-S cells. The lower side of the transwell membrane was coated with increasing amounts of osteopontin (OPN) in the presence or absence of increasing amounts of osteopontin on the upper side of the transwell membrane. Data are expressed as migratory index (cells migrating in response to osteopontin/cells migrating in response to buffer). Values are expressed as mean $\pm$ standard error, numbers in bold are significantly different from control values with $p < 0.01$ or better. D,E) Left panel: Haptotaxis of MH-S cells across polycarbonated filters in response to osteopontin (300 pmoles) bound to the lower surface. In controls wells, coated filters were placed into the Boyden chamber with the coated side up or with both sides coated (compare C)). Right panel: Chemotactic activity was

tested with soluble osteopontin (300 nM) in the lower chamber. Monocyte migration was mainly directional (i.e., the cells responded to a positive gradient of osteopontin, compare A,B)). D) Purified natural osteopontin (nOPN) exerted chemotactic and haptotactic activity for the MH-S monocyte cell line through distinct domains which were differentially inhibited by modulators of integrin binding (GRGDS peptide, anti-integrin β_3 antibody 2C9.G2) or CD44 binding (antibody KM81, clone TIB 241). The antibody concentrations used were saturating in a preliminary experiment as judged by titration of anti-integrin β_3 antibody (haptotactic indices 9.8 at 0 μg, 2.0 at 0.1 μg, 2.2 at 0.2 μg, 2.2 at 0.5 μg) and anti-CD44 (chemotactic indices 13.3 at 0 μg, 4.9 at 0.1 μg, 4.4 at 0.5 μg, 5.1 at 1 μg, 4.3 at 2 μg). E) Recombinant osteopontin (rOPN) was phosphorylated with the indicated kinases as previously described [30,40,41,42]. rOPN (GK), recombinant osteopontin phosphorylated with Golgi kinases isolated for mouse calvarial cells (14 mol of phosphate/mol protein); rOPN (CKII) rOPN phosphorylated with casein kinase II (9 mol phosphate/mol protein); rOPN (CKI) phosphorylated with casein kinase I (11 mol phosphate/mol protein); rOPN (PKA) recombinant osteopontin phosphorylated with cAMP dependent kinase (low incorporation of phosphate); rOPN (PKG) recombinant osteopontin phosphorylated with cGMP dependent protein kinase (3 mol phosphate/mol protein); OPN-NT10k N-terminal 10 kD fragment of osteopontin. Since phosphorylation at any site never reaches 100% the mol phophate/mol protein underestimate the total number of sites phosphorylated. Data are expressed as migratory index (cells migrating in response to osteopontin/cells migrating in response to buffer). Values are expressed as mean $\pm$ standard error, numbers in bold are significantly different from control values with $p < 0.05$ or better. Similar results were obtained with MT-2/1 cells (not shown).

Figure 1: Titration of osteopontin into the peritoneum induces a cellular infiltrate in a dose dependent manner. The numbers reflect induction ratios after 6 h of A) total cells (○) or B) cells with the surface markers Mac-1^+ (▲), $B220^+$ (■) or $CD3^+$ (□) in mice injected with osteopontin versus mice injected with PBS (baseline cell numbers for PBS-injected mice ranged from: 443,000 to $1.1x10^6$ cells total; 224,000 to 596,000 Mac-1^+ cells; 31,300 to 176,000 $B220^+$ cells; 18,100 to 214,100 $CD3^+$ cells). The data points are combined from three experiments. Error bars, where indicated, are mean ± standard error.

Figure 2: Expression of CD44 and integrin β_3 by MH-S and MT-2 cells. The expression levels of CD44 and integrin β_3 were measured by flow cytometry with FITC-conjugated antibodies to these surface receptors (anti-pan-CD44 clone IM7 and 2C9.G2) and an irrelevant control antibody (open peak, not labeled). RT-PCR with primers for the constitutive exons 5 and 16 of CD44 (MT-2 CD44 and MH-S CD44) amplifies a prominent band of about 200 bp in both macrophage cell lines (with these primers, the standard form would yield a product of 135 bp [39]) indicating that standard CD44 is not abundantly expressed. RT-PCR with primers for the exons 5 and v6 (MT-2 v6 and MH-S v6) results in three PCR products, ranging in size from around 200 bp to 400 bp, corroborating the expression of multiple CD44 variants that contain the exon v6. No bands were seen in the no-template controls (H2O CD44 and H2O v6). The two lateral lines indicate the positions of the markers for 194 bp and 300 bp.

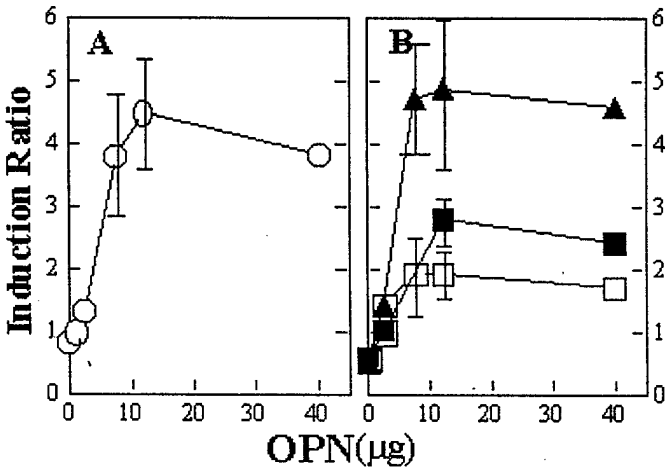
Figure 3: Phosphorylation of osteopontin is required to induce cell spreading. MH-S monocytic cells (10^3/well) or MT-2/1 cells (not shown) were incubated in 96-well plates that had been coated with 10 μg/ml (300 nM) of the indicated ligands for 1 hr before washing, staining

with toluidine blue, and counting. Top panel) Change in cell morphology (1) MH-S cells attached to and spread on immobilized phosphorylated osteopontin (2) MH-S cell bound to unphosphorylated osteopontin do not spread (original magnification 400X). Middle panel) Numbers of cells attached to the indicated ligands in the presence or absence of GRGDS peptide or of the control peptides GRGES or SGRSD. Values are expressed as mean $\pm$ standard error. Bottom panel) Numbers of cells spread on the indicated ligands in the presence or absence of GRGDS. Values are expressed as mean $\pm$ standard error. rOPN = recombinant osteopontin, ~P = native phosphorylated osteopontin, - = no osteopontin, NT = N-terminal thrombin cleavage fragment of osteopontin, CT = C-terminal fragment.

Figure 4: Induction of metalloprotease secretion by phosphorylated but not unphosphorylated osteopontin. MH-S cells were stimulated for 6 hours with either phosphorylated or unphosphorylated osteopontin at a concentration of 10 μg/ml (300 nM) in serum-free defined medium. In order to visualize the secreted metalloproteases, gelatin zymograms were performed. MMP-9 and pre-MMP-9 are both visible in the sample stimulated with natural osteopontin (lane 2) and samples stimulated with phosphorylated recombinant osteopontin (lane 3). Dephosphorylation of osteopontin with acid phosphatase abolishes the stimulatory activity of osteopontin (lane 4). Similarly, recombinant osteopontin has no stimulatory activity (lane 5). The pre-form, but not active MMP9, is stimulated by the N-terminal fragment of osteopontin (lane 6), while the C-terminal fragment of osteopontin has little or no stimulatory activity (lane 7). Control: MH-S cells were incubated with serum-free defined medium (lane 1).

Figure 5: Osteopontin induces secretion of inflammatory mediators from macrophages. Resident peritoneal macrophages, obtained by peritoneal lavage with phosphate-buffered saline were treated with red cell lysis buffer and incubated (10^5 macrophages per 100 μl) for 2 hours. The adherent fraction was incubated with 5 nM osteopontin (OPN = native osteopontin, dpOPN = dephosphorylated native osteopontin, rOPN = recombinant osteopontin, rOPN~P = recombinant osteopontin phosphorylated by Golgi kinases) or 30 ng/ml lipopolysaccharide (LPS). Supernatant IL-1β, IL-6, IL-10, IL-12 and TNFα were assayed at 24 or 48 h post stimulation, with commercial ELISA kits (R&D Systems, Minneapolis, MN). Similar results were obtained with the MH-S cell line.

Figure 6: Model of monocyte regulation by osteopontin. Osteopontin is secreted by activated T-cells. Thrombin cleavage releases the two receptor-binding domains which carry out distinct functions in the cascade of events leading to macrophage attraction and activation. The C-terminal piece exerts chemotactic activity that is phosphorylation independent, leading to attraction of macrophages to the cleavage site and cellular attachment to the osteopontin N-terminal fragment. Phosphorylation-dependent haptotaxis on cross-linked osteopontin or OPN-NT leads to macrophage spreading and activation, including induction of cytokine secretion and release of metalloproteases that can degrade the matrix.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57
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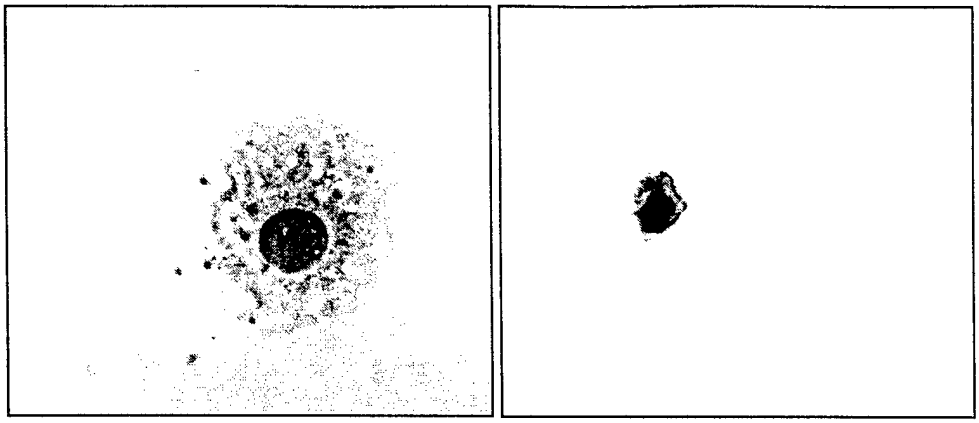

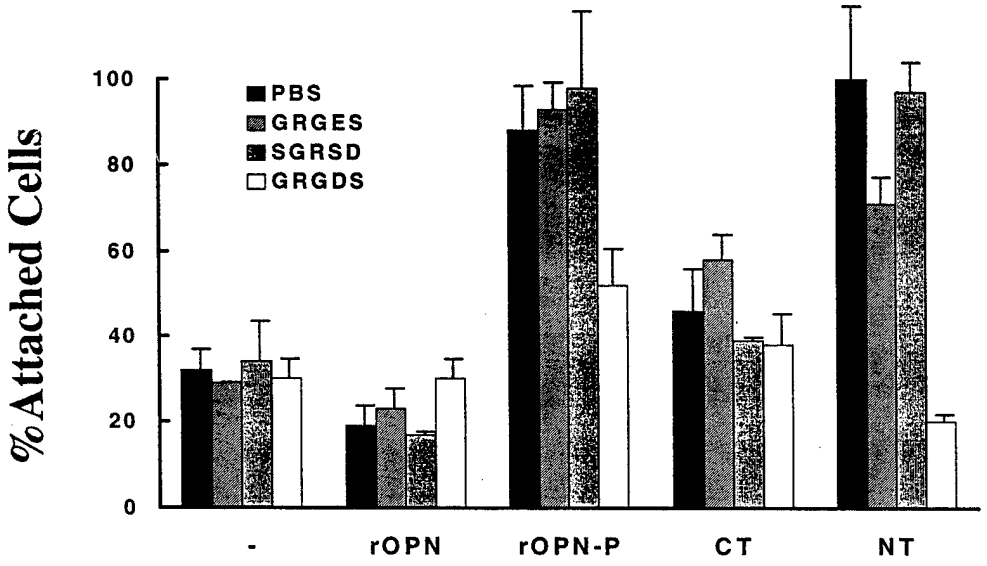
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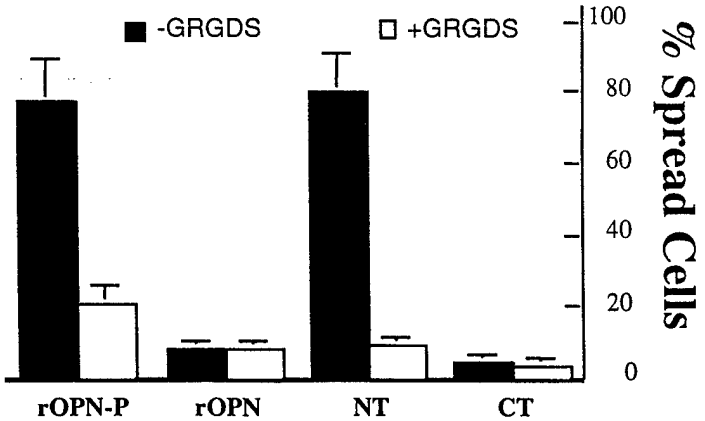
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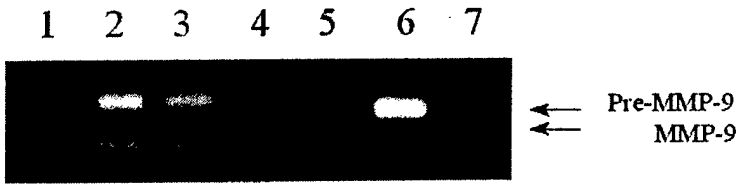
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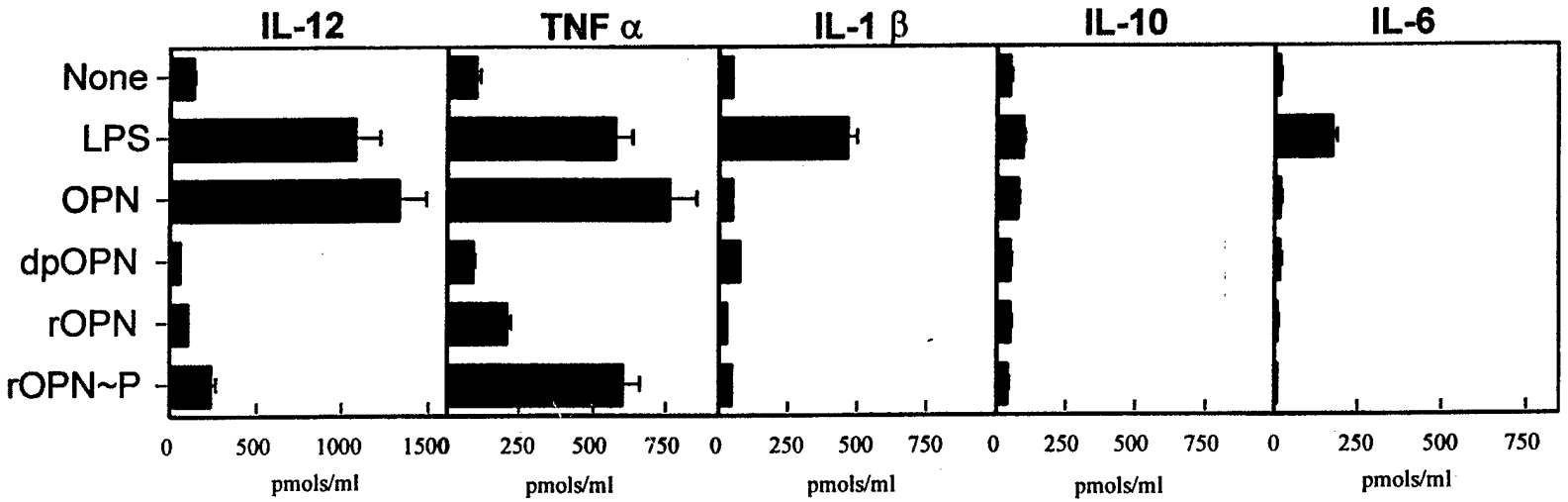
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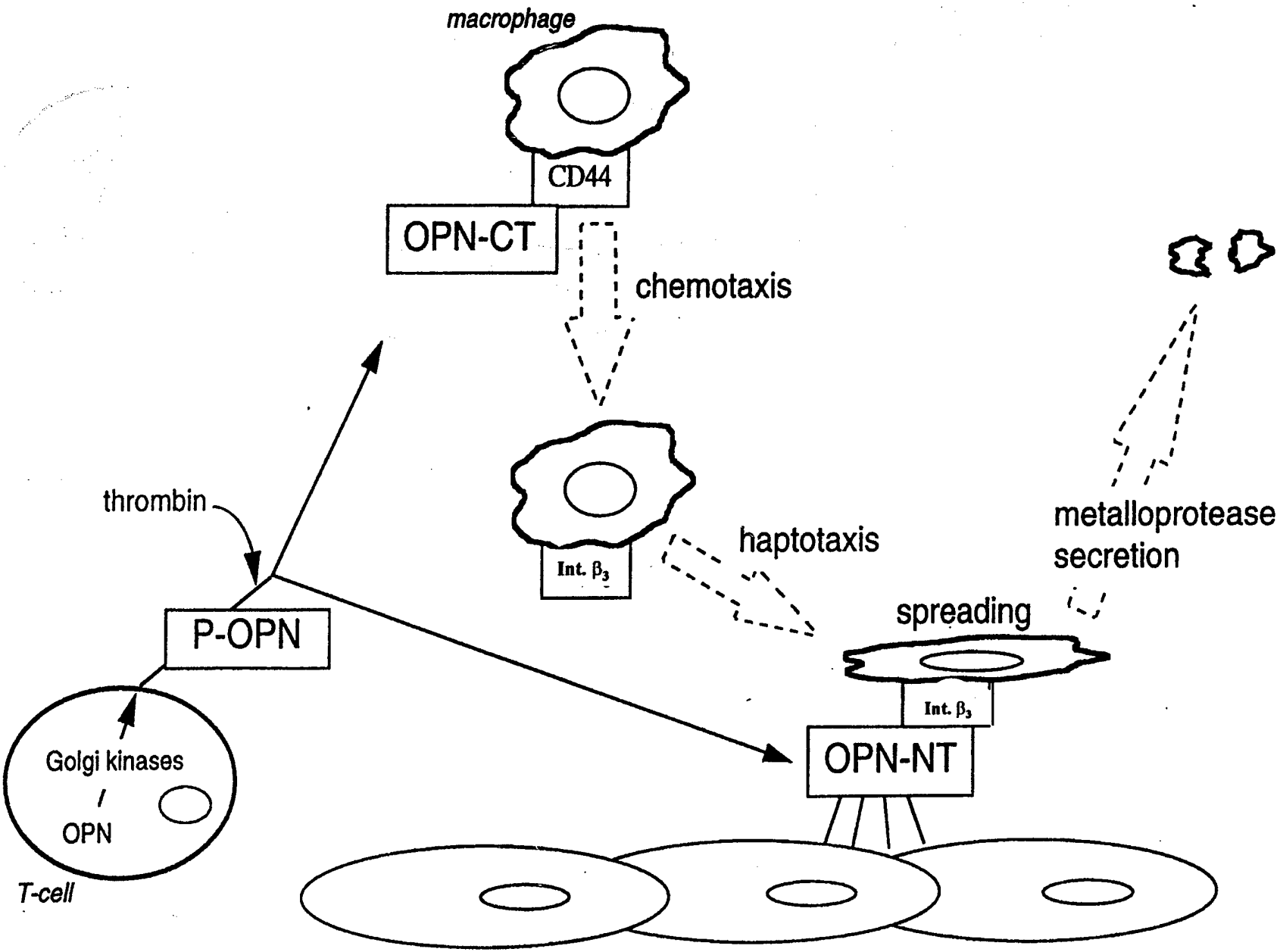
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OPN-NT
Golgi kinases
OPN
T-cell

Phosphorylation of NF-κB Proteins by Cyclic GMP-Dependent Kinase: A Non-Canonical Pathway to NF-κB Activation

Running Title: Phosphorylation of NF-κB by PKG

Bin He[1], Ranjan Sen[3], Georg F. Weber[1,2,+]

[1]Department of Radiation Oncology, New England Medical Center, Boston, MA 02111, USA.

[2]Immunology Program, Sackler School of Graduate Biomedical Research, Tufts University Medical School, Boston, MA 02111, USA.

[3]Rosenstiel Basic Medical Research Center and Department of Biology, Brandeis University, Waltham, MA 02454, USA.

[+] Address correspondence to: Georg F. Weber, Department of Radiation and Cancer Biology, New England Medical Center, NEMC #824, 750 Washington Street, Boston, MA 02111, USA. Phone (617) 636-9013; fax (617) 636-1766. E-mail gweber@lifespan.org.

SUMMARY

Activation of the transcription factor NF-κB may be regulated by various modes of phosphorylation. P65, p49, and p50 are specific substrates for cyclic GMP-dependent protein kinase (PKG). Phosphorylation of p65 by PKG dose-dependently increases its transactivating activity from the NF-κB consensus sequence. Phosphorylation of p50 or p49 mediates a dose-dependent increase in transcriptional activity from a unique C/EBP-associated NF-κB site, but not from the consensus site. These results imply a novel mechanism for PKG-induced gene transcription and they reveal a form of differential regulation of NF-κB activity by phosphorylation of the DNA-binding subunits. They may also bear on ongoing efforts to induce cancer cell apoptosis through activation of PKG. Furthermore, our experiments highlight potentially confounding factors in transient transfection assays with reporter genes. Conventionally, co-transfections are performed with plasmids containing highly active viral promoters, such as CMV. The NF-κB binding sites in the transfected CMV promoters can compete with the NF-κB sites in the relevant reporter constructs, thus suppressing the reporter signal. NF-κB proteins can alter the expression levels of co-transfected genes by transactivation from the CMV promoter. Finally, co-transfected gene products may transactivate the reporter gene, independently of or synergistically with NF-κB. These sources of interference need to be taken into account during the performance of reporter gene experiments.

INTRODUCTION

The enzyme cyclic GMP-dependent kinase (cGK, PKG) is an important mediator of intracellular signal transduction, involved in such diverse processes as the regulation of blood vessel tone, platelet aggregation, and long term potentiation in memory formation (1, 2, 3). Furthermore, PKG may regulate apoptosis positively (4, 5, 6, 7, 8) or negatively (9), possibly depending on other modulating biochemical events (10). Efforts have been made to induce cancer cell apoptosis with sulindac sulfone (exisulind, Aptosyn), which activates PKG (11, 12, 13). Neoplastic ovarian epithelial cells may, however, down-regulate PKG levels (14), possibly contributing to enhanced tumor cell survival. Although PKG – like cyclic AMP-dependent kinase (PKA) – can phosphorylate and activate the transcription factor CREB the connection of PKG to gene expression is incompletely understood. Recently, the localization of the PKG form I in the cytosol and the nucleus was reported (15, 16), suggesting a broader role for PKG in the regulation of gene transcription.

The transcription factor NF-κB (17, 18) mediates a wide range of cellular stress responses. Its biologic roles necessitate a rapid activation of the preformed cytosolic complex, which is typically accomplished by phosphorylation. NF-κB phosphorylation is functionally relevant on three levels. Firstly, it targets the inhibitor I-κB for degradation. Even though phosphorylation of I-κB is not sufficient to dissociate the complexes of I-κB and NF-κB in vivo, phosphorylation on serines 32 or 36 is a prerequisite for I-κB degradation in the ubiquitin-proteasome pathway (19). Secondly, phosphate residues contribute to the processing of the p100 and p105 NF-κB precursor proteins for p50 and p49/p52. A prerequisite for the proteolytic cleavage of p105 is the phosphorylation of serines 894 and 908, which are potential recognition sites for proline-directed serine/threonine kinases, including cyclin-dependent kinases and Erk2 kinase (20). The phosphorylation of the C-

terminal region of p105 may be mediated by cyclic AMP-dependent protein kinase or protein kinase C (21). Finally, direct phosphorylation of the DNA-binding NF-κB subunits can regulate their functions.

Phosphorylation of the DNA-binding NF-κB subunits may modulate DNA binding affinity, transactivation, or the interaction with other regulatory proteins. A PKA recognition sequence within the Rel homology domain, which contains DNA binding sites and nuclear localization signals, has been linked to the transformation of avian spleen cells by *v-rel* and to the cytoplasmic retention of c-Rel in chicken embryo fibroblasts (22). PKA activates NF-κB in a manner that is independent of I-κB phosphorylation, does not impair its interaction with unmodified p105, and induces transactivation (21, 23). This is accomplished through the recruitment of CBP/p300 by phosphorylated p65 (24, 25, 26). Some investigators have not found p65, p50, or p52 to be phosphorylated by PKA or PKC (21, 27). While p65 and p50 have recognition sequences for PKA or PKG, p49 does not have such a consensus site (22, 28), however related sequences are found in all three molecules. Our previous studies (7) suggested that NF-κB may be activated by PKG. Subsequent investigations indicated that p49 and p50 are substrates for the kinase. Here we analyze the mechanisms by which PKG induces NF-κB activation.

EXPERIMETNAL PROCEDURES

Reagents --Recombinant human NF-κB p49 and p50 were obtained from Promega. The α-isozyme of cGMP-dependent protein kinase, purified from bovine lung or recombinant bovine, was purchased from Promega or Calbiochem. The following oligonucleotides were utilized in gel shift reactions after radiolabeling with T4 polynucleotide kinase: NF-κB consensus (Promega, sense 5'-AGT TGA GGG GAC TTT CCC AGG C-3'), OCT1 (Promega, sense 5'-TGT CGA ATG CAA ATC ACT AGA A-3'), H2K (sense 5'-GGA TCC CGG TCG GGG GAT TCC CCA TCT CGG-3'), κ enhancer (sense 5'-AGC AGA GGG GAC TTT CCG AGG C-3'), NF-κB-CRE (a NF-κB site from the CMV promoter sequence, sense 5'-CAA TAG GGA CTT TCC ATT GAC GTC AAT GGG-3'). The custom made oligonucleotides were obtained as single stranded and were annealed to double stranded probes after phosphorylation with T4 polynucleotiode kinase and γ-^{32}P-ATP. Double-stranded poly(dI-dC)·poly(dI-dC) was purchased from Pharmacia. The cGMP-dependent kinase inhibitor Rp-8-pCPT-cGMPS was purchased from Biolog.

Various reporter constructs were used in this study. They included a commercial NF-κB luciferase reporter (pNF-κB-luc, Clontech) that contains four consensus NF-κB sites. A luciferase reporter containing the C-reactive protein promoter (pC/EBP-luc) and a relevant control with a mutated NF-κB p50 binding site (pC/EBP-mP50-luc) were generously provided by Dr. David Samols. We cloned the luciferase reporter gene into the vector pCR3.1, which contains the CMV promoter sequence, to generate a luciferase reporter for CMV promoter activity (pCR3.1-luc).

Kinase reaction--The enzymatic activity of cGMP-dependent kinase was analyzed in kinase reaction buffer (250 mM Mes, pH 6.9, 2 mM EGTA, 5 mM magnesium acetate, 50 mM NaCl, 10 mg/ml BSA, 100 mM dithiothreitol, 2 mM protein kinase A inhibitor peptide) with 1mg/ml Kemptide substrate (LRRASLG) and 1 mM γ-^{32}P-ATP (30 to 40 cpm/pmol). The reaction was

performed in the presence or absence of 200 μM cyclic GMP at room temperature for 3 min. The reaction was terminated by spotting 50 μl onto Whatman P-81 filter paper and immediate immersion in 10 ml 75 mM H_3PO_4 for 2 min. This was followed by 5 washes in 10 ml 75 mM H_3PO_4, air drying, and scintillation counting.

Phosphorylation of PKG substrates (93 ng/μl for recombinant NF-κB p49 and p50; 500 ng/μl for p65) was performed in 100 mM Tris-HCl, pH 7.5, 20 mM sodium chloride, 10 mM dithiothreitol, 2 mM magnesium acetate, 200 μM ATP, at room temperature for 15 minutes. The kinase concentration was 3.5 U/μl, while cyclic GMP was present at 200 μM. MOPS buffer was not used because it is incompatible with the electrophoretic mobility shift assay after transfer. There was no loss of PKG enzymatic activity in Tris buffer at the indicated concentration.

293T cells ($1x10^6$ per 100 mm diameter Petri dish) were transiently transfected with 0.3 μg pFLAG-p49 or pRSV-p65 with $CaCl_2$. 24 hours after transfection, the cells were lysed in 0.5 ml RIPA buffer and precleared with 30 μl 20% protein A agarose beads overnight. 1 μg anti-FLAG or anti-p65 antibodies were added for 2 h at 4°C followed by pulling down with 30 μl 20% protein A agarose beads for an additional 1 h. The agarose beads were pelleted at 14,000 g for 1 min and washed four times in cold RIPA buffer, then two times in detergent-free buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl). Kinase reaction buffer was directly added to the pelleted beads with or without PKG and cGMP as indicated plus 1 μl γ-^{32}P-ATP for 15 min at room temperature. The reaction solutions were then resolved on 8% reducing denaturing SDS-polyacrylamide gel and transferred to PVDF membranes for autoradiographic exposure and Western blotting.

Electrophoretic mobility shift assay--DNA binding was assessed by electrophoretic mobility shift according to standard protocols. The reaction mixture contained 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0, 35 mM NaCl, 50 μg/ml poly(dI-dC)·poly(dI-dC), and 5% glycerol plus labeled

probe. The DNA binding proteins were transferred from the kinase reaction mixture. The reaction was incubated at room temperature for 20 minutes before separation on a native 4% polyacrylamide gel. For supershift, the appropriate antibodies (0.05-0.1 μg) were incubated with the nuclear extracts for 10 min at room temperature before adding DNA binding buffer.

Cloning of relevant gene products--To clone mouse PKG Iα cDNA, frozen mouse kidney tissue was homogenized and total RNA was isolated by using RNeasy mini kit from Qiagen (Valencia, CA) following the manufacturer's protocol. 1 μg total RNA was used for cDNA synthesis with Superscript II RNase H$^-$ reverse transcriptase (Gibco BRL, USA). The coding sequence of PKG Iα was amplified with the primers 5'-AGCATGGGCACCCTGCGGGATTTA-3' and 5'-ATTAGAAGTCTATGTCCCAGCCTGAGTTG-3'. The amplified fragment was cloned into the vector pCR3.1 (Invitrogen Carlsbad, CA) followed by subcloning into the vector pEF6/His B (Invitrogen, Carlsbad, CA). Sequence fidelity and accurate reading frame were verified by DNA sequencing analysis.

Targeted mutations in p65 were generated in positions 276 and 305 by PCR cloning with the Quickchange site directed mutagenesis kit (Stratagene) according to the protocol by the manufacturer. The sense mutagenic oligonucleotides used were 5'-GCGGCGGCCTGCCGACCGGGAGCTCAGT-3' for S276A and 5'-AAACGTAAAAGGGCATATGAGACCTTCAAGAGCATC-3' for T305A (mutations underlined). The accuracy of the mutations was confirmed by DNA sequencing.

P49 was Flag-tagged at the 5'-end by PCR using the primers 5'-CTGCAGCATGGACTACAAGGACGACGATGACAAGGAGAGTTGCTACAACCCAGGTCTG-3' and 5'-GAGAGTTGCTACAACCCAGGTCTG-3' with pRSV-p49 as a template. The

amplified fragment was cloned into the vector pCR3.1 and sequence fidelity was confirmed by DNA sequencing.

Reporter gene assays --293T cells were plated at 1 x 10^6 cells per 100 mm diameter Petri dish and were grown for 24 hours before transfection with $CaCl_2$. The commercial pNF-κB-luc reporter (Clontech, Palo Alto, CA) contains four NF-κB response elements and was used at 0.5 μg per transfection. The common internal transfection standard pRL-SV40 (10 ng per transfection) served as a control for transfection efficiency. Renilla was not used in transfection experiments with the non-commercial reporter constructs because the luminescence intensity is too high compared to the specific readout. 24 hours after transfection, the cells were harvested in 1 ml reporter lysis buffer (Promega) and dual luciferase reporter assays were performed following the protocol provided by the manufacturer. Lysates were diluted 1:40 and 10 μl were used for measurement in a luminometer (Turner Designs TD-20/20). In reporter gene experiments without Renilla, luminescene was measured in 40 μg (total protein) of lysate. All assays were done at least in triplicates. The protein concentrations were determined by the BCA protein assay reagent kit (Pierce). As confirmation of protein expression, 20 μg of the same lysates were also used for separation on 8% SDS-polyacrylamide gels followed by Western blotting on PVDF membranes. PKG kinase activity in the lysates was confirmed by phosphorylation of the standard substrate LRRASLG ("kemptide") where indicated.

Western blotting--Cells were lysed in RIPA (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% sodium dodecyl sulfate) or NTEN buffer (20 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5% NP40) containing 1 mM phenyl-methyl-sulfonyl-fluoride (PMSF), 10 μg/ml pepstatin, and 1 mM dithiothreitol. The lysates were centrifuged at 15 000 g for 5 min and the protein concentration was determined in the supernatants. 20 μg total protein were

resolved on reducing denaturing SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were probed with appropriate antibodies followed by horseradish peroxidase-conjugated secondary antibodies and development using enhanced chemiluminescence. Anti-p65 antibody (C-terminus, rabbit polyclonal), anti-p50 anitbody (rabbit polyclonal), and anti-p52 antibody (rabbit polyclonal, used to detect p49) were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-PKG antibody (C-terminal, aa 657-671, rabbit polyclonal) was purchased from Calbiochem (La Jolla, CA). Reprobing of the membranes with anti-tubulin antibody (Sigma) served as additional loading control.

Cells --Reporter assays were performed by transient transfections of 293T cells. O3 is a $CD4^+$ T-helper cell clone derived from BALB/c mice after in vitro selection for proliferation to ovalbumin in association with BALB/c antigen-presenting cells (29). O3 cells express $V_\beta 6$ and respond to conventional antigen (ovalbumin) and to the retroviral superantigen MTV-7 ($Mls\text{-}1^a$) (30). The AF3.G7 hybridoma was generated by fusing beef insulin immune C57BL/6 lymph node cells with the BW5147 thymoma line. It bears $V_\beta 6$ and $V_\alpha 3.2$ and responds to MTV-7 according to IL-2 production (31). After stimulation by conventional antigen or superantigen, the T-cells were obtained by passage through Cell-ect^{TM} columns (Biotex Laboratories Inc., Edmonton, Alberta, Canada) for the preparation of nuclear extracts. As control for activation, measurement of ^3H-thymidine incorporation by O3 cells after stimulation with mitomycin C-treated LBB cells as antigen-presenting cells was performed as described previously (30).

RESULTS

NF-κB may be activated by PKG in T-lymphocytes--T-lymphocytes respond to engagement of their antigen receptor by conventional antigen peptide with proliferation. We have previously described an alternative signal transduction pathway, associated with the T-cell antigen receptor, that is induced by superantigen and leads to activation of PKG and programmed cell death (7). We compared the induction of NF-κB in the T-cell clone O3 following stimulation by conventional antigen or retroviral superantigen. In electrophoretic mobility shift assays measuring the binding to a NF-κB consensus probe, both bands were induced by the conventional antigen. In contrast, predominantly the lower band was induced by stimulation with superantigen (Fig. 1), which activates signal transduction involving PKG. This was not due to quantitative differences in stimulation because both modes of T-cell activation induced comparable levels of tritiated thymidine incorporation in the same experiment. Treatment of nuclear extracts from resting O3 T-cell clones or AF3.G7 T-cell hybridoma cells with PKG and cGMP induced DNA binding of the lower band to a NF-κB consensus oligonucleotide. A similar induction was seen in AF3.G7 cytosol and this was inhibitable by increasing amounts of the competing PKG substrate peptide GRTGRRNSI ("PKI substrate"). We therefore set out to investigate the potential role of PKG in the induction of NF-κB.

P49, p50, and p65 are substrates for cyclic GMP-dependent kinase--NF-κB p49, p50, and p65 are phosphorylated by cyclic GMP-dependent kinase and the phosphorylation levels are enhanced by addition of cyclic GMP. The substrate peptide GRTGRRNSI ("PKI substrate"), but not the control peptide GRTGRRNAI, inhibits phosphorylation of p49 or p50 as well as autophosphorylation of cGMP-dependent kinase in a dose-dependent manner. Similar observations

were made for the inhibition of p65 phosphorylation by GRTGRRNSI (see Fig. 8B). The kinase reactions on p49 and p50 are also inhibited by the cGMP-dependent kinase inhibitor Rp-8-pCPT-cGMPS at a high concentration. Consistent with the competitive function of the compound, inhibition is complete in the absence of cGMP but partial in the presence of cGMP (Fig. 2A-C). There is substantial substrate overlap between PKA and PKG. Cyclic AMP-dependent kinase also phosphorylates p50 with comparable efficiency, but p49 is phosphorylated strongly by cGMP kinase and only very weakly by PKA (data not shown).

We expressed p49 and p65 by transient transfection in 293T cells. P65 was immunoprecipitated with a specific antibody and p49 was immunoprecipitated with an antibody to Flag-tag. The pulled-down proteins were phosphorylated by PKG in vitro. Both NF-κB subunits incorporated radioactive phosphate (Fig. 2D). In vivo binding between p65 or p49 and PKG was further confirmed by co-immunoprecipitation of the kinase with an antibody to either p65 or Flag-tag (for p49) after co-transfection of the kinase with either of the NF-κB proteins (Fig. 2E).

Phosphorylation of NF-κB proteins increases their transactivating activity from distinct NF-κB sites-- We asked whether PKG can alter the transactivating activity of p65 as judged by luciferase assays with a commercial NF-κB luciferase reporter that contains four consensus NF-κB sites. Co-transfection of increasing amounts of PKG enhanced dose-dependently the transactivating activity of transfected p65. The results were consistent with the protein expression levels and kinase activities in the cell lysates (Fig. 3A). Therefore, phosphorylation of p65 by PKG may increase its transactivating ability.

To determine the specificity of the PKG-dependent induction of p65 transactivating activity we performed co-transfection experiments with other reporters that contain NF-κB binding sites.

Consistent with earlier reports (32, 33, 34), p65 does not transactivate a non-consensus motif associated with C/EBP, which is found in the C-reactive protein promoter (Fig. 3B). In both cases, the lack of transactivation is not overcome by PKG.

The NF-κB protein p50 contains a DNA binding domain, but no transactivation domain. Nevertheless, transactivation may be observed after transfection of p50 into cells, presumably due to its binding to endogenous interaction partners. Those include most prominently p65, but also Bcl-3 (35). In addition, p50-dependent transactivation may occur from a non-consensus site in conjunction with C/EBP (32, 33). We used the non-consensus reporter construct in transient co-transfection assays. No reporter activity was induced by PKG, whereas p50 dose-dependently increased luciferase activity (data not shown). Co-transfection of PKG with low amounts of p50 (0.3 μg DNA) dose-dependently enhanced its transactivating activity (Fig. 4A) consistent with the increased affinity of p50 to this DNA sequence after phosphorylation by PKG (see below). In all transactivation experiments, comparable results were obtained with murine p50 (data not shown). P49 and p50 have similar DNA binding characteristics. We found p49 to also transactivate from the non-consensus NF-κB site in a manner that could be increased dose-dependently by co-transfected PKG (Fig. 4B).

We then tested whether p50 or p49 transactivate a commercial luciferase reporter that contains four NF-κB consensus sites and whether transactivation under these conditions might be modulated by PKG. Luciferase activity was not induced by transfection of p49 or p50. Furthermore, co-transfection of p50 or p49 with increasing amounts of PKG did not lead to measurable transactivation from the commercial luciferase reporter (Fig. 4C).

When we expressed the genes of interest under the control of a CMV promoter we noted that the transfected PKG kinase activity and protein levels were substantially increased by co-

transfection of p50, but not of p65. We reasoned that three NF-κB sites in this promoter sequence likely accounted for the induction. Therefore, PKG and p50 were re-cloned into pEF6/HisB and pEF6/HisA respectively. The luciferase reporter gene was sub-cloned into the CMV promoter-containing vector pCR3.1 to generate a suitable reporter construct for measuring transactivation from the CMV promoter. Transfection of either PKG or p50 dose-dependently induced CMV promoter-dependent reporter activity. The former may have acted through four CREB sites in the CMV enhancer sequence. The transactivation from the CMV promoter reporter induced by co-transfected PKG and p50 was additive, rather than synergistic, which suggested that they act mutually independently through distinct binding sites (Fig. 5).

In sum, the transactivation experiments using reporter assays indicated that phosphorylation by PKG can enhance the transcriptional activity of the DNA binding NF-κB subunits. In contrast, PKG does not confer transactivating potential from non-cognate NF-κB sites. Because the proteins p49, p50, and p65 direct transcription from distinct DNA sequences, their phosphorylation by PKG enhances their differential effects.

Phosphorylation of p50 by PKG directly impacts its DNA binding affinity--Changes in transactivating activity may reflect alterations in DNA binding affinity. Cyclic GMP-dependent kinase is inactive under standard gel shift assay conditions. Conversely, gel shift assays cannot be performed in the PKG reaction buffer. We therefore adjusted the kinase reaction buffer so that we could phosphorylate NF-κB proteins and then transfer an aliquot to the standard DNA binding buffer for analysis of phosphorylation-dependent changes in the affinity to DNA. After transfer of p49 or p50 from kinase assays to gel shift reaction mixtures, phosphorylation by cGMP-dependent kinase did not affect the binding of the p49 or p50 homodimers to the H2K probe, to which these

proteins already have high affinity without being phosphorylated. Binding of p50 to the NF-κB consensus sequence or to the non-consensus NF-κB-C/EBP or NF-κB-CRE sequences is induced by PKG in vitro. After co-transfection, the binding affinity by nuclear extracts to the same probes is similarly induced and the specificity of the main DNA binding band from transfectants of p50 or p50 plus PKG was confirmed by supershift (Fig. 6).

The subcellular localization of NF-κB subunits is not affected by phosphorylation with PKG--NF-κB is an inducible transcription factor, which is retained in the cytosol in resting cells. It was therefore possible that the phosphorylation of DNA binding subunits might affect their nuclear import as would be reflected in their subcellular distribution. Transfection of increasing amounts of PKG did not alter the relative fractions of co-transfected p49, p50, or p65 in cytosols and nuclei. Although not definitive, these observations made phosphorylation-induced changes in the transport and half-lives of these NF-κB subunits unlikely (Fig. 7).

The phosphorylation of p65 occurs on non-consensus sites--P65 is a substrate for cyclic AMP-dependent kinase (24), an enzyme, whose substrate specificity is similar to PKG. Likely recognition sites for both enzymes are in positions serine 276 and threonine 305 on p65, and serine 276 has been demonstrated to be phosphorylated by cyclic AMP-dependent kinase. We mutated both candidate phosphorylation sites. The transactivating activity was diminished moderately by the mutation T305A and substantially by S276A, however, co-transfection of PKG led to dose-dependent increases in reporter activity in all cases. Consistent with this observation, synthetic peptides covering the threonine 305 (EKRKRTYETF) or the serine 276 (MQLRRPSDRE) did not incorporate radioactive phosphate during incubation with the kinase, whereas the standard substrate

peptide LRRASLG ("Kemptide") did. We also phosphorylated bacterial recombinant p65-His (36) with PKG in vitro and were able to compete the kinase reaction with the substrate peptide GRTGRRNSI, but not with the peptides covering threonine 305 or serine 276 (Fig. 8). These results support the hypothesis that PKG phosphorylates p65 in positions distinct from the amino acids 276 and 305.

DISCUSSION

The incorporation of phosphate into p49 or p50 depends on PKG, is enhanced by cGMP, can be competed by a PKG substrate peptide but not by a control peptide, and is inhibitable by a cGMP analog with inhibition being more efficient in the absence of cGMP than in its presence. The sum of these observations indicate that NF-κB p49 and p50 are specific substrates for PKG. Similarly, phosphorylation of p65 was shown to depend on the kinase, be increased in the presence of cGMP, and to be competed out by substrate peptide. Cyclic GMP-dependent kinase, like cyclic AMP-dependent kinase, has a preference for the phosphorylation of serines or threonines found close to at least two consecutive N-terminal basic residues. The standard PKG recognition site is (R,K)(R,K)X(S,T). It is important to note, however, that there are a number of exceptions to this rule. The p50 precursor p100 has PKG recognition sites in positions 335 and 940 (Genbank accession numbers M57999 or NM_003998). Although p49 (Genbank accession number A57034) does not contain any PKG consensus sites, there are five similar sites with the sequence X(R,K)X(S,T) in p49 (aa 76, 195, 201, 231, 430) that may conceivably serve as candidate recognition motifs. The consensus sites for PKG in p65 are at positions 276 and 305 (Genbank accession number M62399). The phosphorylation of amino acid 276 by PKA is of major importance for p65 transcriptional activity. Remarkably, our experiments indicate that PKG phosphorylates p65 in positions other than the two consensus sites. The phosphorylation of p65 by PKA and PKG on distinct residues implies multiple modes of regulation of p65 function. It also indicates that the position, in which phosphorylation occurs, is an important determinant for transactivation.

Recognition sequences for NF-κB have been identified in the promoters of a large number of genes. Beside the consensus sequence, additional NF-κB binding sites are known and may differ in

their affinities to various subunits of the transcription factor. The release of cytosolic p65/p50 heterodimers into the nucleus is by itself insufficient to differentiate among the numerous NF-κB promoter sequences. Our results suggest a mechanism of differential transactivation of various NF-κB dependent genes by phosphorylation of the DNA-binding subunits. Phosphorylation of p65 by PKG induces transcription from the consensus sequence, whereas phosphorylation of p50 or p49 induces transcription from a C/EBP-associated non-consensus site but not from the consensus sequence. PKG 1 has been shown to be in the cytosol as well as in the nucleus (15). It may therefore exert its regulatory role before or after translocation of p65 and p50 to the nucleus.

Beside the well studied induction of CREB, there has been little evidence for the activation of transcription factors by cGMP-dependent kinase. Transfection of BHK cells with PKG causes transactivation of the *c-fos* promoter, mediated most notably through SRE, FAP, and CRE (37). The connection of PKG and NF-κB may have broad implications since a pathway that involves PLA_2, hydroxyl radical, guanylate cyclase, and PKG has been mapped to T-cell signal transduction leading to apoptosis (7) as well as to long term potentiation in the central nervous system (3). Cellular NF-κB activation by H_2O_2 has been shown to involve protein phosphorylation and therefore to be mediated indirectly via induction of a kinase (21). The cGMP-dependent kinase may thus provide a link for the NF-κB activation by reactive oxygen species because the activity of guanylate cyclase can be regulated by redox reactions.

Conventionally, co-transfections are performed with plasmids containing highly active viral promoters, such as CMV. Among various samples within the same experiment, the total amount of DNA transfected is typically kept constant by balancing with the CMV containing vector. Our studies have indicated that the NF-κB binding sites in the transfected CMV promoters can compete with the NF-κB sites in the reporter constructs. This leads to a profound reduction of the reporter

signal, in our experiments up to 80%. Furthermore, NF-κB proteins such as p50 can alter the expression levels of co-transfected genes by transactivation from one of the NF-κB sites in the CMV promoter. The present investigations have revealed the potential for a reciprocal influence between the co-transfected kinase and p50 under these circumstances, which interfered with the quantitation of phosphorylation induced changes in p50-dependent transactivation. This was further complicated by four CREB sites that mediated PKG-dependent transactivation. One of these CREB sequences is adjacent to a NF-κB site in the CMV promoter and may have contributed to the p50 induced transactivation. This has become plausible through the recent demonstration that activated NF-κB may interact with the CRE binding protein (25, 26). Alternatively, C/EBP could bind to the CRE site (38, 39) and synergize with p50 in inducing transactivation. For accurate measurements, we were forced to re-clone both PKG and NF-κB genes into a vector that is not sensitive to either set of transcription factors. Although reporter assays are currently often done with constructs that contain CMV promoter sequences, these sources of interference need to be taken into account during the performance of these types of assays.

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ABREVIATIONS

PBS	phosphate-buffered saline
PKA	cyclic AMP-dependent kinase
PKG	cyclic GMP-dependent kinase
PLA_2	phospholipase A_2
U	unit

REFERENCES

1. Hofmann, F., Ammendola, A., and Schlossmann, J. (2000) *J. Cell. Sci.* **113,** 1671-1676
2. Ruth, P. (1999) *Pharmacol. Ther.* **82,** 355-372
3. Weber, G. F. (1999) *Neurosci. Biobehav. Rev.* **23,** 1079-1086
4. Chiche, J. D., Schlutsmeyer, S. M., Bloch, D. B., de la Monte, S. M., Roberts, J. D. Jr, Filippov, G., Janssens, S. P., Rosenzweig, A., and Bloch, K. D. (1998) *J. Biol. Chem.* **273,** 34263-34271
5. Loweth, A. C., Williams, G. T., Scarpello, J. H., and Morgan, N. G. (1997) *FEBS Lett.* **400,** 285-288.
6. Taimor, G., Hofstaetter, B., and Piper, H. M. (2000) *Cardiovasc. Res.* **45,** 588-594
7. Weber, G. F., Abromson-Leeman, S., and Cantor, H. (1995) *Immunity* **2,** 363-372
8. Weber, G. F. (2002) in *Calcium Homeostasis and Signaling in Aging* (Mattson MP, ed.). In press
9. Takuma, K., Phuagphong, P., Lee, E., Mori, K., Baba, A., and Matsuda, T. (2001) *J. Biol. Chem.* **276,** 48093-48099.
10. Canals, S., Casarejos, M. J., de Bernardo, S., Rodriguez-Martin, E., and Mena, M. A. (2001) *J. Neurochem.* **79,** 1183-1195
11. Liu, L., Li, H., Underwood, T., Lloyd, M., David, M., Sperl, G., Pamukcu, R., and Thompson, W. J. (2001) *J. Pharmacol. Exp. Ther.* **299,** 583-592
12. Piazza, G. A., Thompson, W. J., Pamukcu, R., Alila, H. W., Whitehead, C. M., Liu, L., Fetter, J. R., Gresh, W. E. Jr, Klein-Szanto, A. J., Farnell, D.R., Eto, I., and Grubbs, C. J. (2001) *Cancer Res.* **61,** 3961-3968

13. Thompson, W. J., Piazza, G. A., Li, H. , Liu, L., Fetter, J., Zhu, B., Sperl, G., Ahnen, D., and Pamukcu, R. (2000) *Cancer Res.* **60,** 3338-3342

14. Wong, A. S., Kim, S.O., Leung, P. C., Auersperg, N., and Pelech, S. L. (2001) *Gynecol. Oncol.* **82,** 305-311

15. Gudi, T., Lohmann, S. M., and Pilz, R. B.(1997) *Mol. Cell. Biol.* **17,** 5244-5254

16. Gudi, T., Casteel, D. E., Vinson, C., Boss, G. R., and Pilz, R. B. (2000) *Oncogene* **19,** 6324-6333

17. Sen, R., and Baltimore, D. (1986) *Cell* **46,** 705-716

18. Sen, R., and Baltimore, D. (1986) *Cell* **47,** 921-928

19. Brown, K., Gerstberger, S., Carlson, L., Franzoso, G., and Siebenlist, U. (1995). *Science* **267,** 1485-1488

20. Fujimoto, K., Yasuda, H., Sato, Y., and Yamamoto, K. (1995) *Gene* **165,** 183-189

21. Naumann, M., and Scheidereit, C. (1994) *EMBO J.* **13,** 4597-4607

22. Mosialos, G., Hamer, P., Capobianco, A. J., Laursen, R. A., and Gilmore, T. D. (1991) *Mol. Cell. Biol.* **11,** 5867-5877

23. Ghosh, S., and Baltimore, D. (1990) *Nature* **344,** 678-682

24. Zhong, H., SuYang, H., Erdjument-Bromage, H., Tempst, P., and Ghosh, S. (1997) *Cell* **89,** 413-424

25. Zhong, H., Voll, and R. E., Ghosh, S. (1998) *Mol. Cell* **1,** 661-671

26. Zhong, H., May, M. J., Jimi, E., and Ghosh, S. (2002) *Mol. Cell* **9,** 625-636

27. Hayashi, T., Sekine, T., and Okamoto, T. (1993) *J. Biol. Chem.* **268,** 26790-26795.

28. Schmid, R. M., Perkins, N.D., Duckett, C. S., Andrews, P. C., and Nabel, G. J. (1991) *Nature* **352,** 733-736

29. Friedman, S., Sillcocks, D., and Cantor, H. (1987) *Immunogenetics* **26,** 193-203

30. Weber, G. F., and Cantor, H. (1994) *J. Immunol.* **152,** 4433-4443

31. Spinella, D. G., Hansen, T. H., Walsh, W. D., Behlke, M. A., Tillinghast, J. P., Chou, H. S., Whiteley, P. J., Kapp, J. A., Pierce, C. W., Shevach, E. M., and Loh, D. Y.(1987) *J. Immunol.* **138,** 3991-3995

32. Cha-Molstad, H., Agrawal, A., Zhang, D., Samols, D., and Kushner, I. (2000) *J. Immunol.* **165,** 4592-4597

33. Agrawal, A., Cha-Molstad, H., Samols, D., and Kushner, I. (2001) *J. Immunol.* **166,** 2378-2384

34. Proesch, S., Heine, A-K., Volk, H-D., and Krueger, D. H. (2001) *J. Biol. Chem.* **276,** 40712-40720

35. Bours, V., Franzoso, G., Azarenko, V., Park, S., Kanno, T., Brown, K., and Siebenlist, U. (1993) *Cell* **72,** 729-739

36. Kim, T. K., and Maniatis, T. (1997) *Mol. Cell* **1**,119-129

37. Gudi, T., Huvar, I., Meinecke, M., Lohmann, S.M., Boss, G.R., and Pilz, R.B. (1996) *J. Biol. Chem.* **271,** 4597-4600.

38. Park, E. A., Roesler, W.J., Liu, J., Klemm, D. J., Gurney, A.L., Thatcher, J. D., Shuman, J., Friedman, A., and Hanson, R. W. (1990) *Mol. Cell. Biol.* **10,** 6264-6272

39. Bakker, O., and Parker, M. G. (1991) *Nucleic Acids* **19,** 1213-1217

LEGENDS TO TABLES AND FIGURES

FIG. 1. **Correlation between PKG activity and NF-κB induction in T-lymphocyte activation.** Left panel, top) Nuclear extracts from the AF3.G7 hybridoma or the O3 clone were phosphorylated in vitro by PKG plus cGMP followed by analysis of DNA binding to an oligonucleotide containing the H2K sequence in gel shift assays. The nuclear extract from O3 cells that had been treated with plate-bound anti-CD3ε antibody served as a positive control for induction of the faster migrating NF-κB band. Left panel, bottom) The induction by PKG plus cGMP of DNA binding by AF3.G7 cytosol is inhibitable by addition of high concentrations of a competing PKG substrate peptide, GRTGRRNSI ("PKI substrate"). In addition, cGMP and PKG did not affect Octamer-1 binding in the same experiment (not shown). Right panel) Time course of NF-κB induction after activation of O3 T-cell clones. O3 cells were stimulated by the conventional antigen ovalbumin (OVA) or retroviral superantigen (MTV-7) for 0, 2, or 4 hours. The T-cells were selected for preparation of nuclear extracts, which were then subjected to incubation with a ^{32}P-labelled NF-κB consensus probe followed by electrophoretic mobility shift assay. The relative intensity of the slower and faster migrating bands after stimulation with MTV or OVA was quantitated by densitometric measurement. The relative density units for the lower band are O3 7.0, OVA 2 h 9.8, OVA 4 h 12.8, MTV 2 h 7.9, MTV 4 h 9.7. The relative density units for the upper band are O3 6.9, OVA 2 h 10.5, OVA 4 h 13.3, MTV 2 h 5.5, MTV 4 h 6.7. Comparable levels of T-cell stimulation were confirmed by tritiated thymidine incorporation.

FIG.2. **The NF-κB proteins p49, p50, and p65 are substrates for cGMP-dependent kinase.** A) Substrate phosphorylation depends on the presence of PKG and is enhanced by the addition of cGMP, whereas cGMP in the absence of the kinase does not mediate measurable incorporation of

phosphate. Autophosphorylation of PKG is represented as the upper band on all gels and reflects a specificity control for effects on the enzyme. B) Phosphorylation of p49 or p50 by PKG (as well as PKG autophosphorylation) is reversible by titration of a competing substrate peptide for the kinase (GRTGRRNSI), but not a control peptide with a mutated serine (GRTGRRNAI). C) An analog of cGMP, Rp-8-pCPT-cGMPS, which can act as an inhibitor of PKG, reverses the enzymatic phosphorylation of p49 or p50. Consistent with the competition for binding to the kinase between cGMP and Rp-8-pCPT-cGMPS, the inhibition is more complete in the absence of cGMP than in its presence. Autophosphorylation of PKG serves as a positive control for kinase activity. D) 293T cells were transiently transfected with p65 or FLAG-tagged p49. The cells were lysed in RIPA buffer and the transfected molecules were pulled down by antibodies to the p65 or Flag. Kinase reaction buffer plus γ-^{32}P-ATP was directly added to the pelleted beads with or without PKG and cGMP for 15 min at room temperature. The reaction mixtures were analyzed by autoradiography and Western blotting. E) 293T cells were transfected with vector or p65, or were co-transfected with NF-κB p65 plus PKG. Alternatively, p49 was transfected with or without PKG. The cells were lysed in NTEN buffer. After preclearing, immunoprecipitation was performed with anti-p65 antibody or anti-Flag-tag antibody (for pull-down of p49). The immunoprecipitates and 5 % of the input were resolved on SDS-PAGE and the resulting Western blot was probed with antibodies to p65, to p52 (recognizes p49), and to PKG.

FIG.3. **Phosporylation of p65 by PKG increases its transactivating activity from consensus sites but not from a non-consensus NF-κB reporter**. A) 293T cells were transiently transfected with 300 ng pRSV-p65 and increasing amounts of pEF6/HisB-PKG as indicated. Transactivation from the co-transfected pNF-κB-luc reporter (500 ng) was measured by luciferase activity. The

values are normalized to luminescence induced by 10 ng co-transfected Renilla construct pRL-SV40 (not shown) and the results (fold induction) are presented as mean ± standard deviation of three samples (middle panel). The protein expression of the transfected molecules correlated with the amounts of DNA introduced into the cells, while tubulin (used as a loading control) remained constant (bottom panel). The PKG activity in 20 μg of cell lysates, as judged by phosphorylation of LRRASLG peptide ("kemptide") in vitro, reflected the amounts of PKG transfected. The kinase activity is indicated as mean ± standard deviation (top panel). B) 293T cells were transiently co-transfected with pC/EBP-wt-luc reporter (1 μg) and pRSV-p65 (0.3 μg) with or without 3 μg pEF6/HisB-PKG. Transfection of 0.5 μg pRSV-p50 with pC/EBP-wt-luc reporter (1 μg) served as a positive control. 24 hours after transfection, the cells were harvested in reporter lysis buffer. 40 μg of lysate samples were used for luciferase assays by luminometer and the values obtained for the vector control group were normalized to 1. The results represent mean ± standard deviation of triplicate samples (top panel). 20 μg of lysates were used for Western blotting to confirm the expression levels of the transfected proteins (bottom panel).

FIG. 4. **Transactivation by p50/p49 from a non-consensus sequence is enhanced by PKG.** A) Transactivation by transfected p50 of a luciferase reporter gene containing a non-consensus NF-κB site that overlaps with a C/EBP site (sequence in top panel). 293T cells were transiently co-transfected with 1 μg pC/EBP-wt-luc reporter (or the control construct pC/EBP-mp50-luc, in which the p50 binding site is mutated, represented as pC/EBP (mP50)) with 0.5 μg pRSV-p50 and the indicated amounts of pEF6/HisB-PKG. 24 hours after transfection, the cells were harvested in reporter lysis buffer and 40 μg lysate samples were used for luciferase assays. The results, measured as fold induction, are presented as mean ± standard deviation of three samples

and the values obtained for the vector control group have been normalized to 1. The transcriptional activity is induced dose-dependently by co-transfection of increasing doses of PKG (third panel from top). 20 μg of the lysates were analyzed for PKG kinase activity (second panel), while another 20 μg of lysates were used for Western blotting to confirm the protein expression levels (fourth panel). PKG activity in cell lysate and Western blotting for the transfected molecules served as transfection controls. B) PKG enhances p49 mediated transactivation from the C/EBP-associated non-consensus site. 293T cells were transiently co-transfected with 1 μg pC/EBP-wt-luc reporter and 0.5 μg pRSV-p49 plus increasing amounts of pEF6/HisB-PKG for analysis of luciferase reporter gene activity. The results, measured as fold induction, are presented as mean ± standard deviation of three samples and the values obtained for the vector control group are normalized to 1. Western blotting confirmed the expression levels of the transfected molecules (bottom panel). C) Neither p49 (top panel) nor p50 (bottom panel) transactivate from the commercial NF-κB reporter gene containing four consensus sites. 293T cells were transiently co-transfected with 0.5 μg pNF-κB-luc plus 0.3 μg p49 or 0.3 μg pRSV-p50 plus increasing amounts of pEF6/HisB-PKG. 10 ng of the Renilla construct pRL-SV40 was also co-transfected to normalize the data for transfection efficiency. Transactivation by p65 (0.3 μg pRSV-p65) in the same experiment is shown as a control. Expression of the transfected proteins was confirmed by Western blotting.

FIG.5. **Reciprocal influence between p50 and PKG in the transactivation of CMV sequences.** Top panel) The CMV promoter sequence, which is part of the pCR3.1 vector, contains three NF-κB binding sites (bold underlined sequences) and four CRE sites (bold italic sequences). Bottom left panel) pRSV-p50 was transiently co-transfected with pCR3.1-PKG. 24 hours after

transfection, the cells were harvested in RIPA buffer. PKG kinase activity was assayed on 20 μg cell lysates, while protein expression levels were measured by Western blotting on another 20 μg of the same cell lysates. Bottom right panel) The luciferase gene was cut from the pNF-κB-luc reporter by Hind III and Xba I, and sub-cloned into the pCR3.1 vector, which contains the CMV promoter (designated as pCR3.1-luc). 293T cells were transiently transfected with 5 ng of pCR3.1-luc plus increasing amounts of pRSV-p50 or pEF6/HisB-PKG, individually or together. The resulting luciferase activity was assayed and the data are plotted as fold induction over baseline.

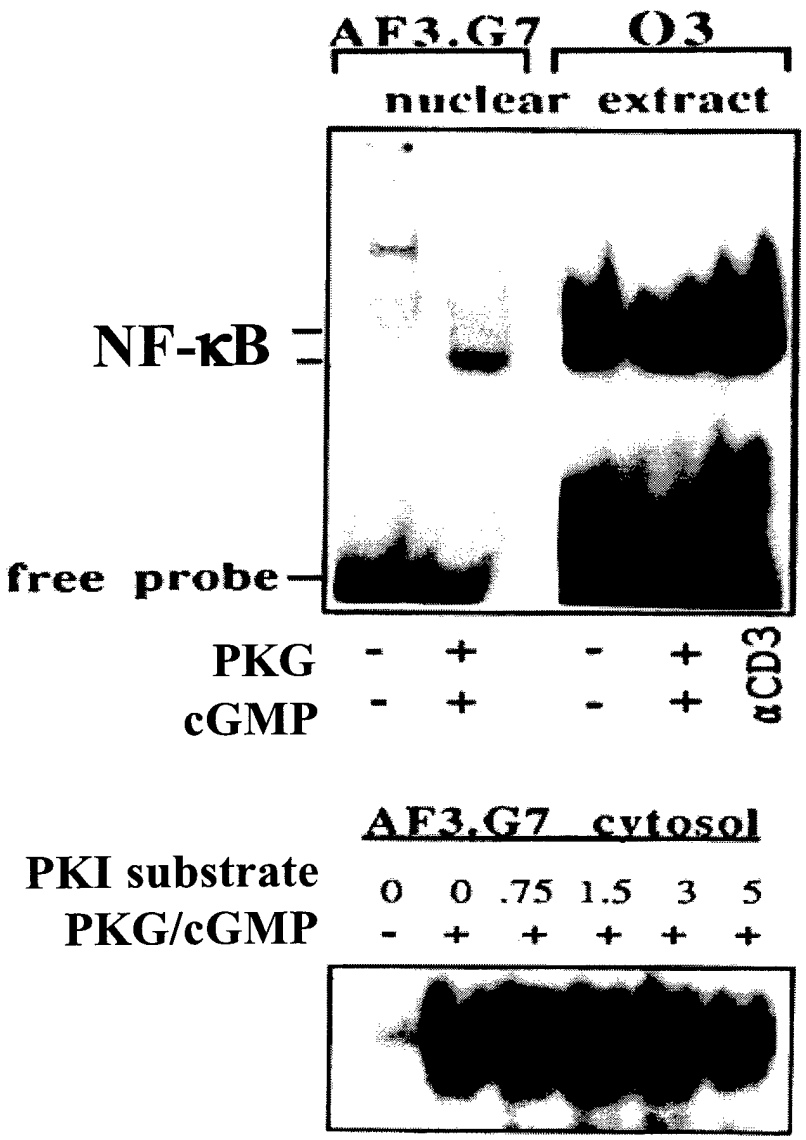
FIG.6. **PKG dependent phosphorylation of p50 induces its affinity to oligonucleotides that represent NF-κB recognition sequences.** P50 binding affinity is induced by PKG to the NF-κB consensus sequence, to the non-consensus sequence (κB-C/EBP), and to a CRE-proximal sequence contained in the CMV promoter (κB-CRE), but not to the κ enhancer sequence or to the H2K sequence. Left panel) 25 ng recombinant p50 per sample were phosphorylated at room temperature for 15 min by 1 U purified PKG with or without 200 μM cGMP in total volume of 10 μl. The reaction mixtures were transferred to DNA binding buffer and incubated for additional 20 min with the indicated ^{32}P-labeled probes. The reactants were resolved on native 4% polyacrylamide gels and exposed to autoradiography film. Right panel) 293T cells were transiently transfected with 0.3 μg pRSV-p50 with or without 3 μg pEF6/HisB-PKG. 24 hours after transfection, the cells were harvested, washed in PBS, and nuclear extracts were prepared. 10 μg of nuclear protein was used for electrophoretic mobility shift assay with radiolabeled NF-κB consensus or OCT1 oligonucleotides (top), or with radiolabeled κB-CRE or κB-C/EBP oligonucleotides (bottom). The identity of the major DNA-binding band was confirmed by supershift with 50 ng

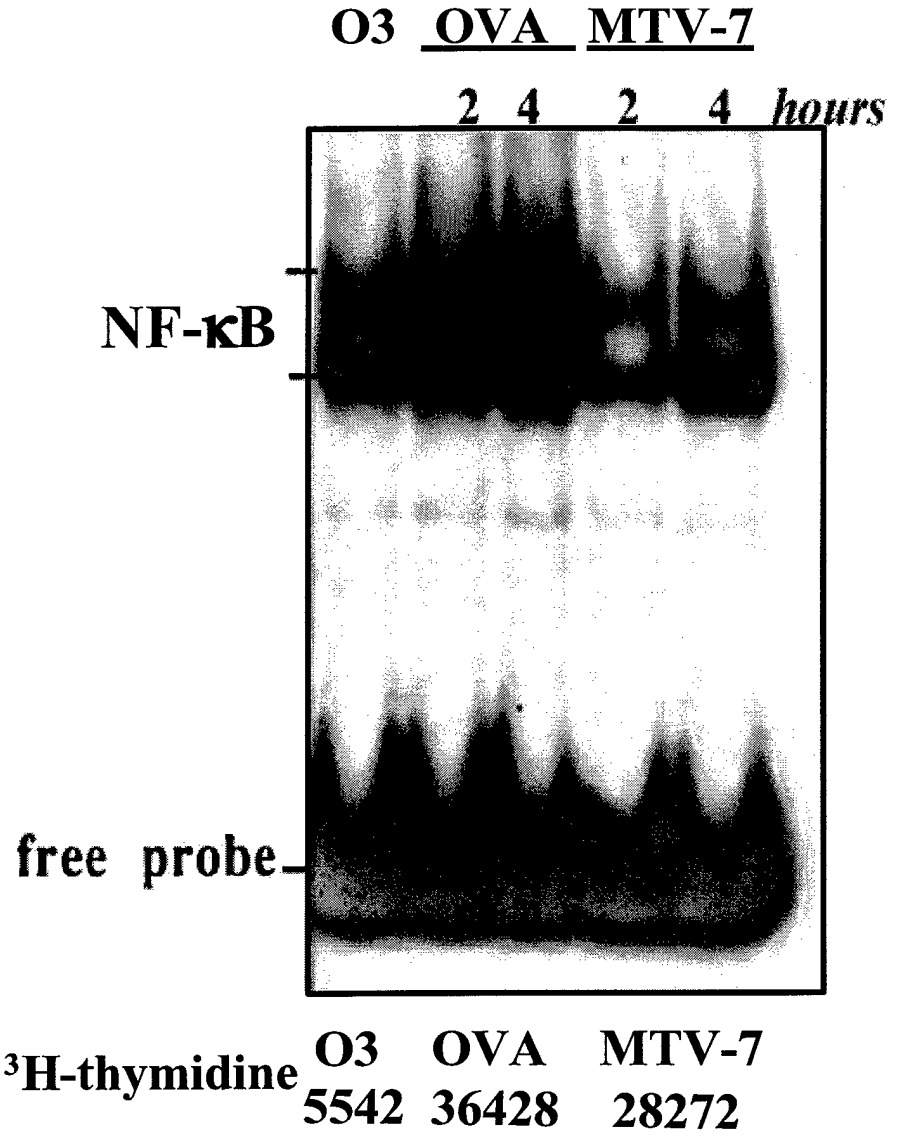
anit-p50 antibody, added to the nuclear extracts at room temperature for 10 min before DNA binding (bottom).

FIG.7. **The subcellular distribution of NF-κB proteins is not affected by PKG.** 293T cells were transiently co-transfected with 0.3 μg pRSV-65 (top panel), pRSV-50 (middle panel) or pRSV-p49 (lower panel) plus increasing amounts of pEF6/HisB-PKG. 24 hours after transfection, the cells were harvested and washed once with PBS for preparation of nuclear extracts and cytosols. The fractions, at 10 μg protein per lane, were resolved on reducing denaturing 8% polyacrylamide gels, transferred to PVDF membranes, and probed with specific antibodies to the indicated proteins.

FIG. 8. **The phosphorylation of p65 by PKG does not occur on the consensus recognition sites.** A) 10 μg of the synthetic peptides T305p65, S276p65, or Kemptide were incubated with or without 1 U PKG plus 200 μM cGMP plus 1 mM γ-^{32}P-ATP in total volume of 100 μl for 3 min at room temperature. One half of the reaction volume was spotted onto filter paper and the reaction was stopped by washes in phosphoric acid. The levels of peptide phosphorylation were determined by scintillation counting and the results are presented as mean values ± standard deviation of three samples. B) 10 μg/ml recombinant p65 was phosphorylated by 1 U PKG in kinase reaction buffer at room temperature for 15 min, in the presence or absence of increasing concentrations of the synthetic peptides GRTGRRNSI ("PKI substrate"), or EKRKRTYETF (T305p65), or MQLRRPSDRE (S276p65). The extent of p65 phosphorylation and PKG autophosphorylation were determined by resolution on reducing denaturing 8% SDS-polyacrylamide gel followed by autoradiography. C) 293T cells were transiently co-transfected with 0.5 μg pNF-κB-luc and 0.3 μg pRSV-p65 or its mutants T305A or S276A plus increasing

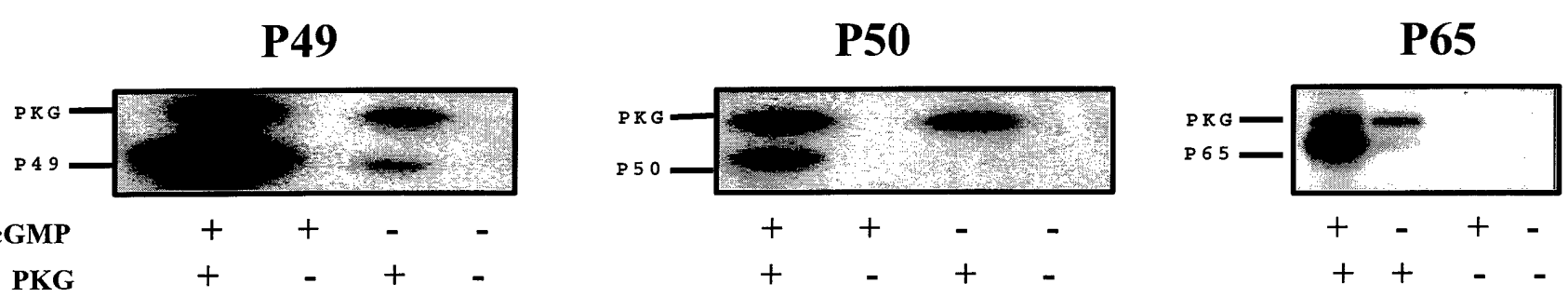
amount of pEF6/HisB-PKG. Co-transfected 10 ng of the Renilla construct pRL-SV40 served as a control for transfection efficiency. 24 hours after transfection, the cells were harvested in reporter lysis buffer and 10 μl of 1:40 diluted lysates were assayed for luciferase activity. The data are presented as fold induction with the values obtained from the vector (pEF6/HisB) transfected cells normalized to 1. The inset shows the PKG-dose dependent increase in transactivation by p65S276A on an adjusted scale.



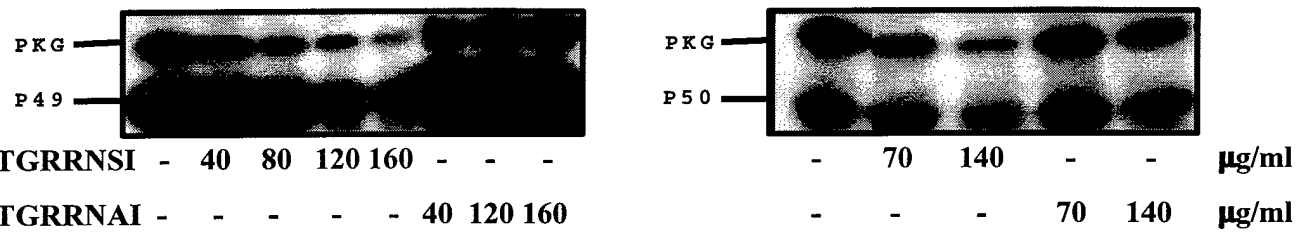


^{3}H-thymidine	O3	OVA	MTV-7
	5542	36428	28272

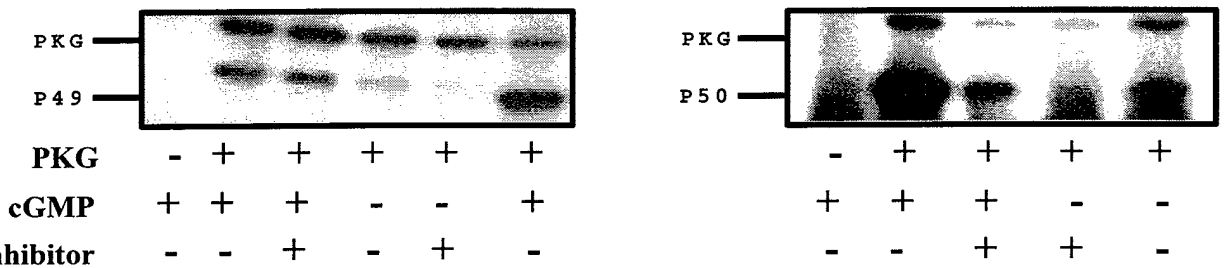
A


P49
P50
P65
PKG
P49
P50
P65
cGMP + + - - + + - - + - + -
PKG + - + - + - + - + + - -

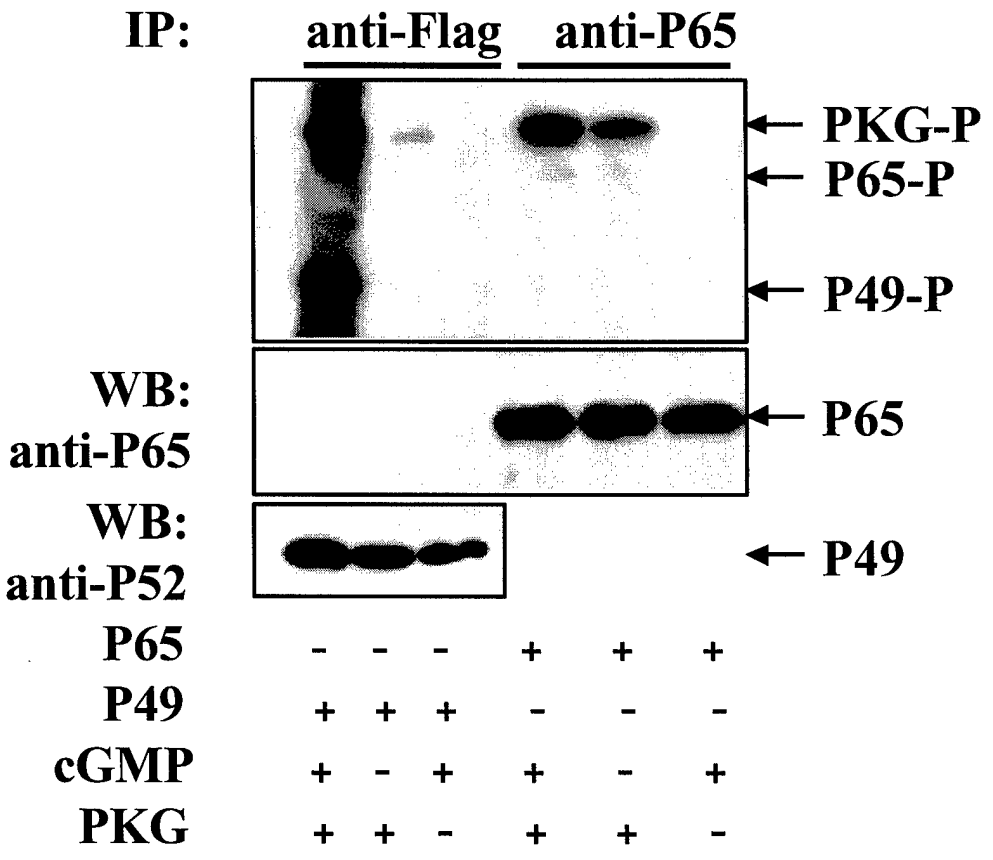
B


PKG
P49
P50
GRTGRRNSI - 40 80 120 160 - - -
GRTGRRNAI - - - - - 40 120 160
- 70 140 - - µg/ml
- - - 70 140 µg/ml

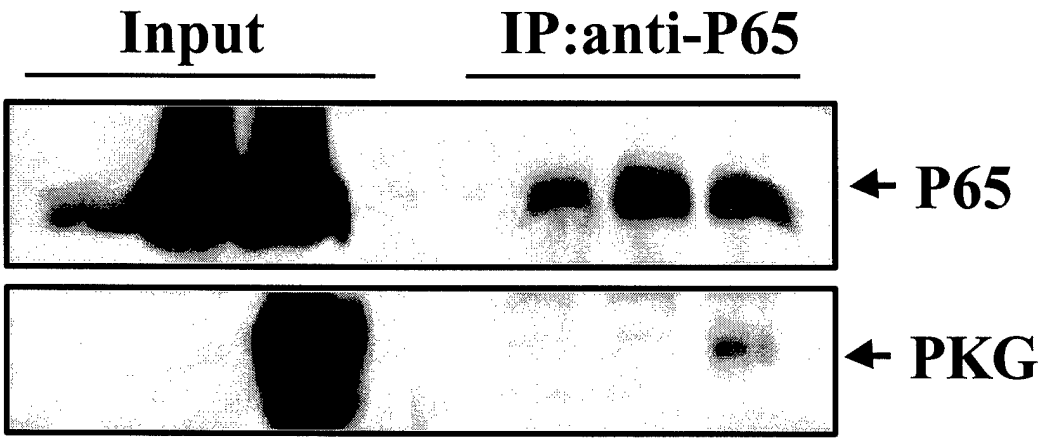
C


PKG
P49
P50
PKG - + + + + +
cGMP + + + - - +
inhibitor - - + - + -
- + + + +
+ + + - -
- - + + -

D

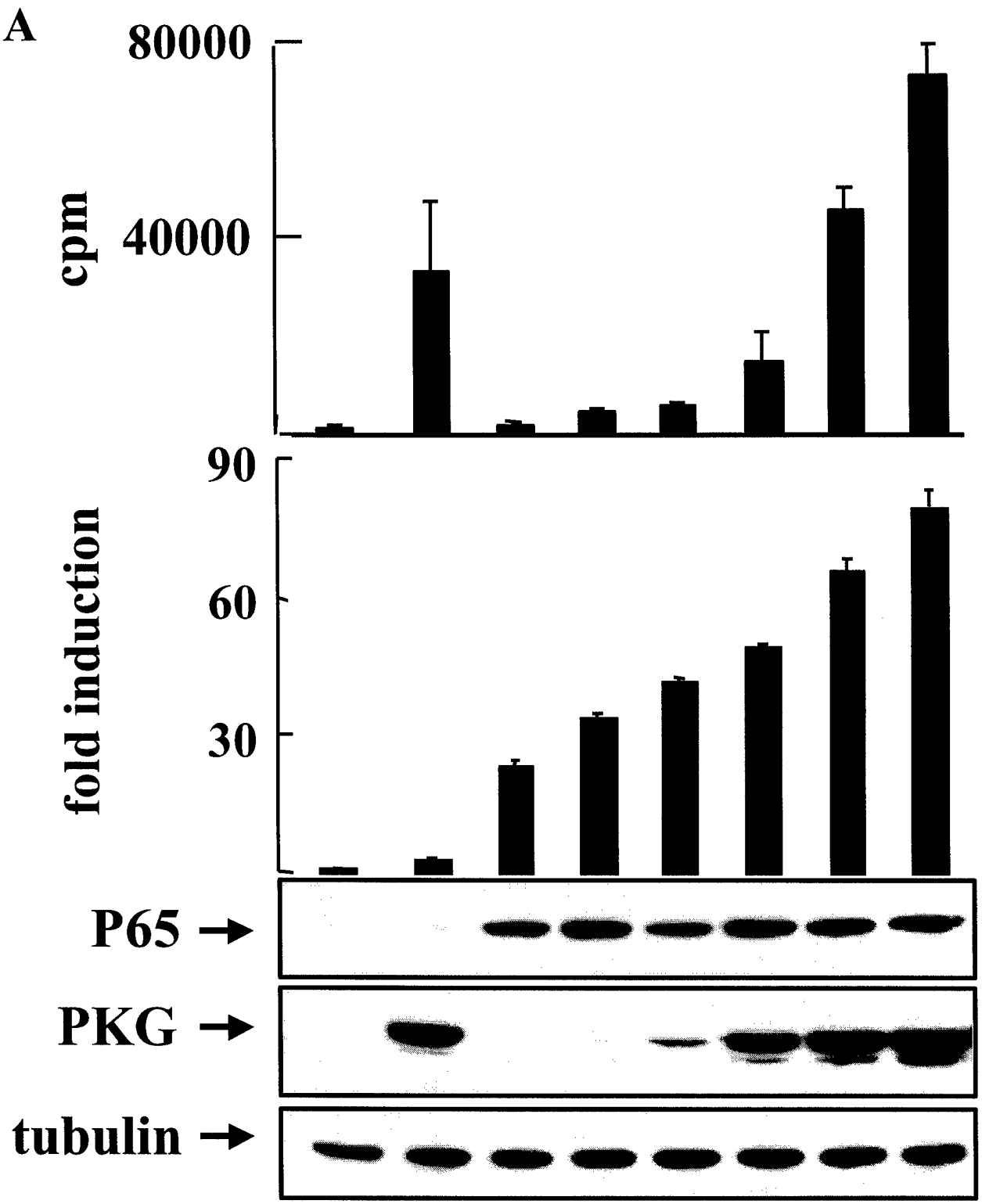

IP:
anti-Flag
anti-P65
PKG-P
P65-P
P49-P
WB:
anti-P65
P65
WB:
anti-P52
P49
P65 - - - + + +
P49 + + + - - -
cGMP + - + + - +
PKG + + - + + -

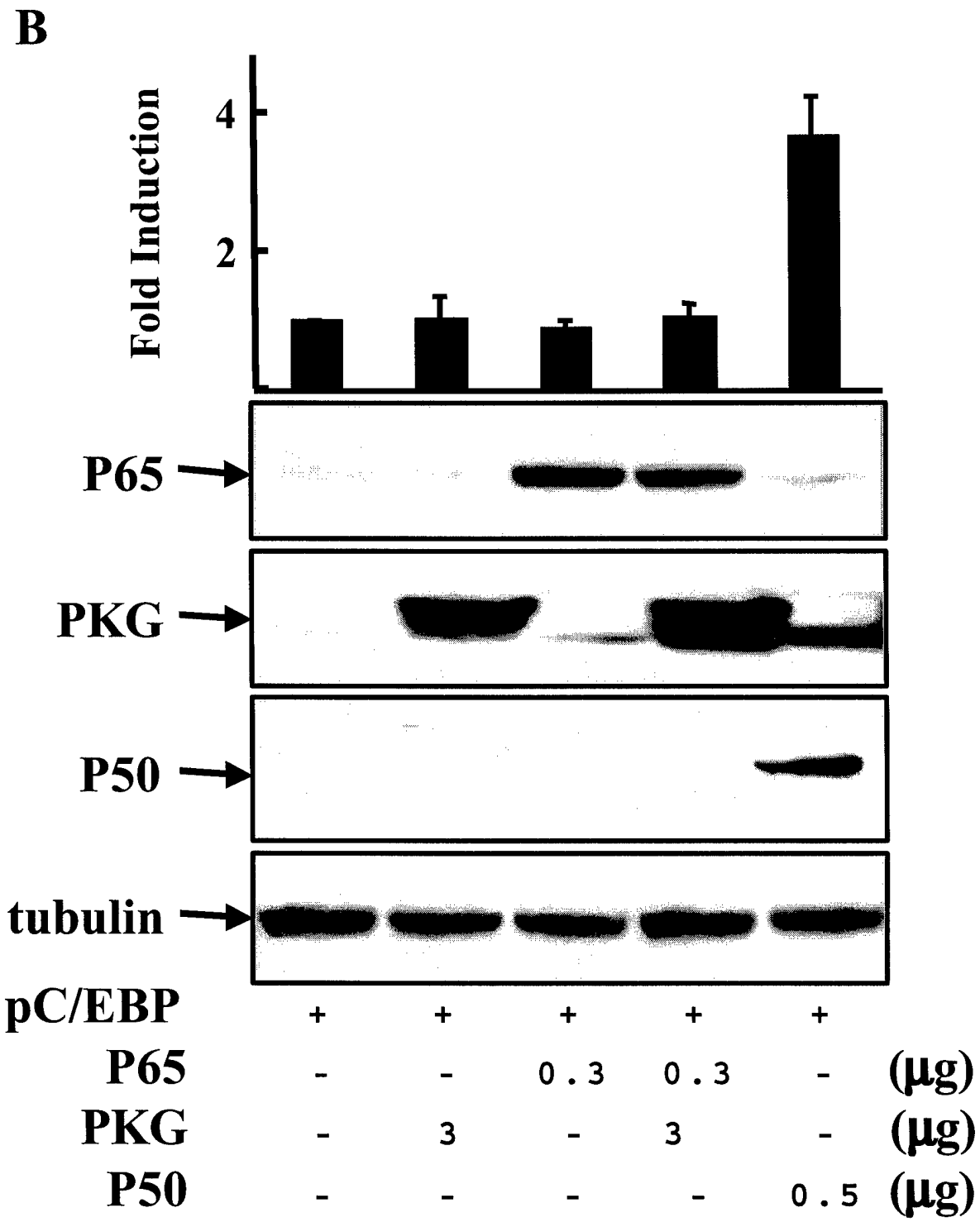
E



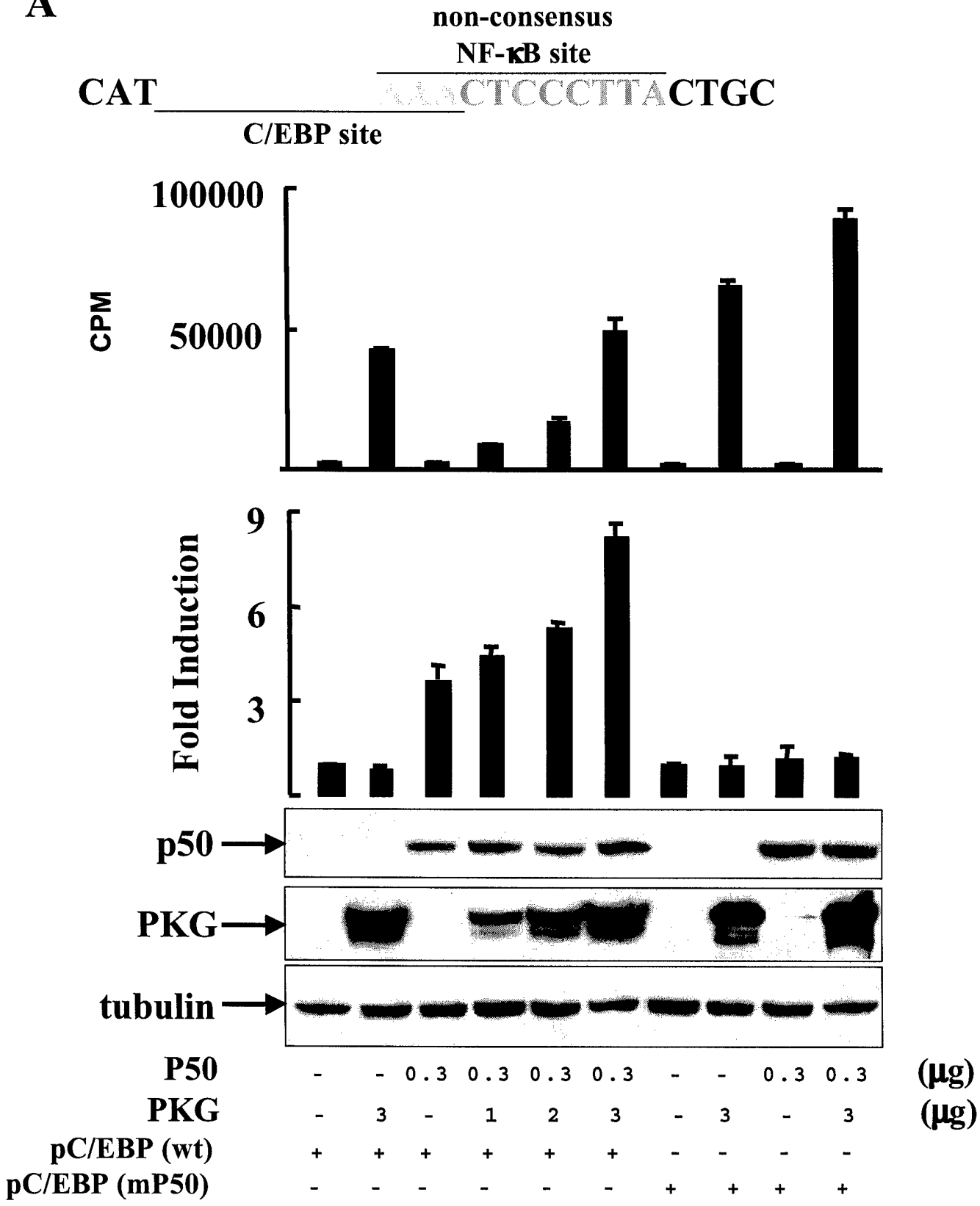
P65	-	0.3	0.3		-	0.3	0.3	µg
PKG	-	-	3		-	-	3	µg

P49	-	0.3	0.3		-	0.3	0.3	µg
PKG	-	-	3		-	-	3	µg

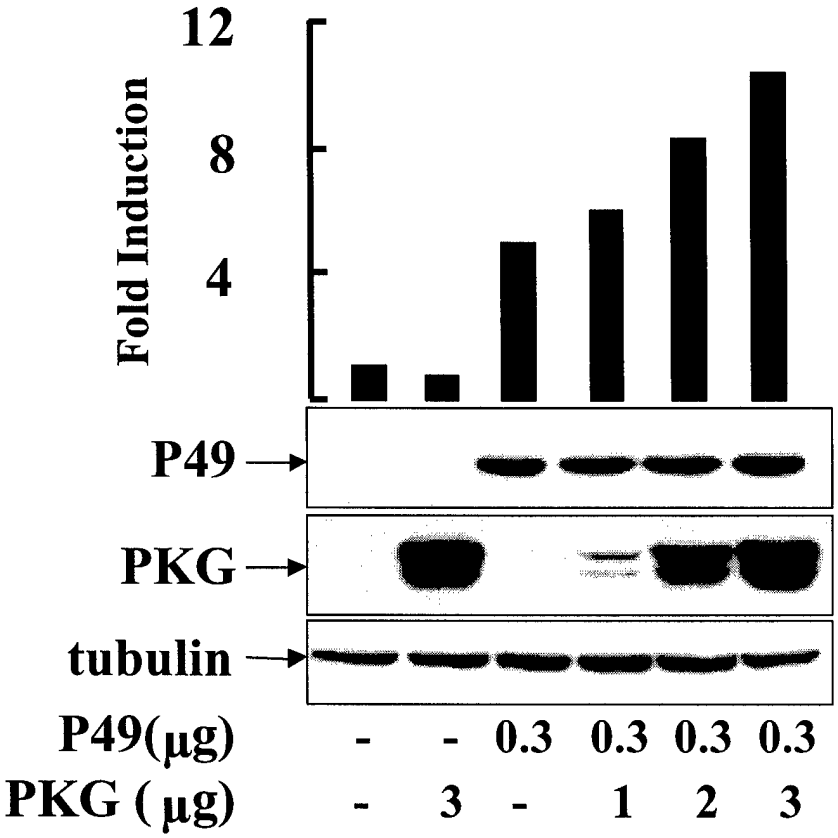

A
80000
40000
cpm
90
60
30
fold induction
P65
PKG
tubulin
P65 (μg) - - 0.3 0.3 0.3 0.3 0.3 0.3
PKG (μg) - 3 - 0.5 1 2 3 4

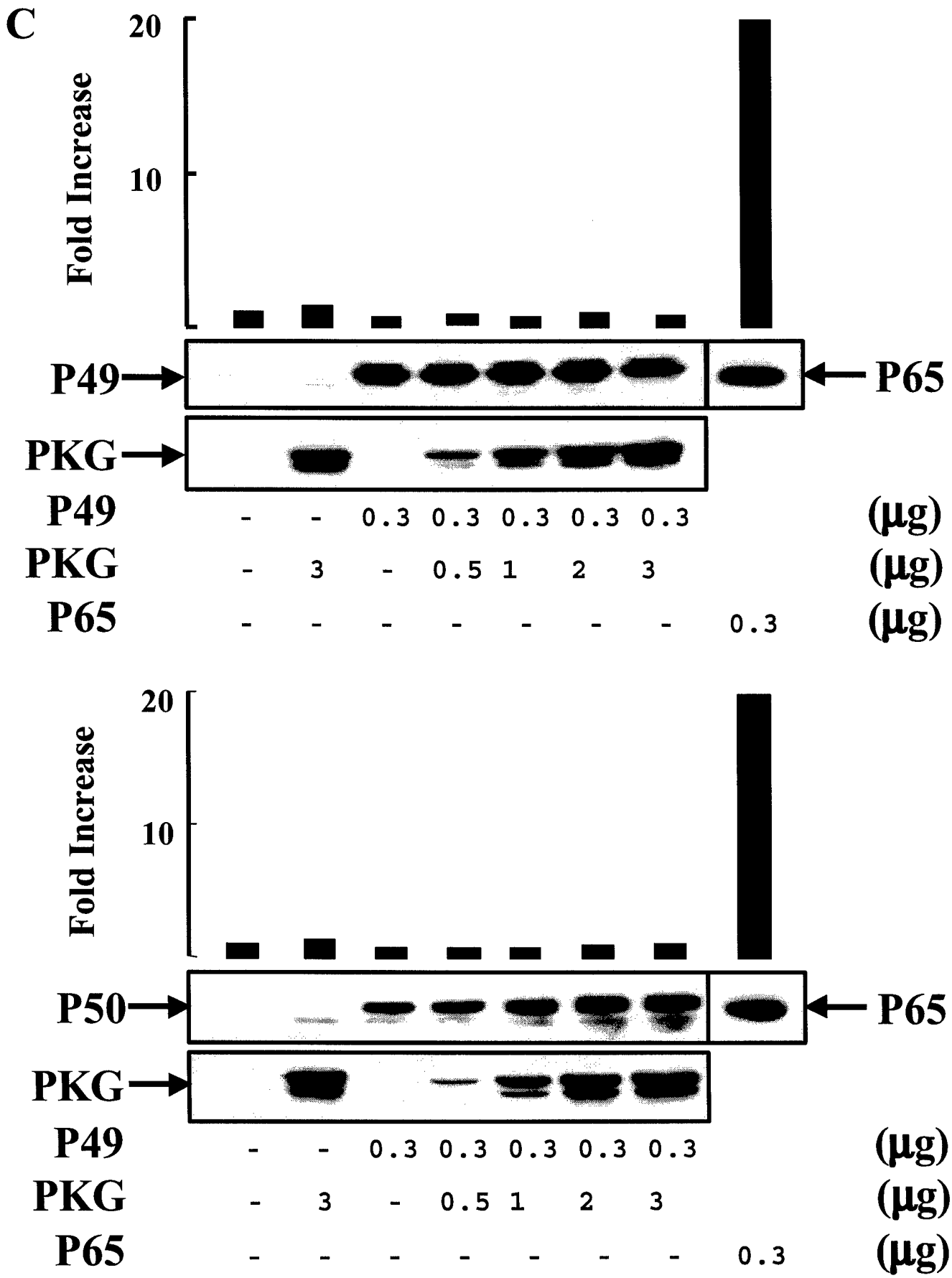

B
Fold Induction
4
2
P65
PKG
P50
tubulin
pC/EBP + + + + +
P65 - - 0.3 0.3 - (μg)
PKG - 3 - 3 - (μg)
P50 - - - - 0.5 (μg)

A


non-consensus
NF-κB site
CAT
CTCCCTTACTGC
C/EBP site
100000
50000
CPM
9
6
3
Fold Induction
p50
PKG
tubulin
P50 - - 0.3 0.3 0.3 0.3 - - 0.3 0.3 (μg)
PKG - 3 - 1 2 3 - 3 - 3 (μg)
pC/EBP (wt) + + + + + + - - - -
pC/EBP (mP50) - - - - - - + + + +

B


12
8
4
Fold Induction
P49
PKG
tubulin
P49(μg) - - 0.3 0.3 0.3 0.3
PKG (μg) - 3 - 1 2 3


C
20
10
Fold Increase
P49
P65
PKG
P49 - - 0.3 0.3 0.3 0.3 0.3 (μg)
PKG - 3 - 0.5 1 2 3 (μg)
P65 - - - - - - - 0.3 (μg)
20
10
Fold Increase
P50
P65
PKG
P49 - - 0.3 0.3 0.3 0.3 0.3 (μg)
PKG - 3 - 0.5 1 2 3 (μg)
P65 - - - - - - - 0.3 (μg)

5' GCGCGCGTTG ACATTGATTA TTGACTAGTT ATTAATAGTA ATCAATTACG GGGTCATTAG
TTCATAGCCC ATATATGGAG TTCCGCGTTA CATAACTTAC GGTAAATGGC CCGCCTGGCT
GACCGCCCAA CGACCCCCGC CCATTGACGT CAATAATGAC GTATGTTCCC ATAGTAACGC
CAATAGGGAC TTTCCATTGA CGTCAATGGG TGGACTATTT ACGGTAAACT GCCCACTTGG
CAGTACATCA AGTGTATCAT ATGCCAAGTA CGCCCCCTAT TGACGTCAAT GACGGTAAAT
GGCCCGCCTG GCATTATGCC CAGTACATGA CCTTATGGGA CTTTCCTACT TGGCAGTACA
TCTACGTATT AGTCATCGCT ATTACCATGG TGATGCGGTT TTGGCAGTAC ATCAATGGGC
GTGGATAGCG GTTTGACTCA CGGGGATTTC CAAGTCTCCA CCCCATTGAC GTCAATGGGA
GTTTGTTTTG GCACCAAAAT CAACGGGACT TTCCAAAATG TCGTAACAAC TCCGCCCCAT
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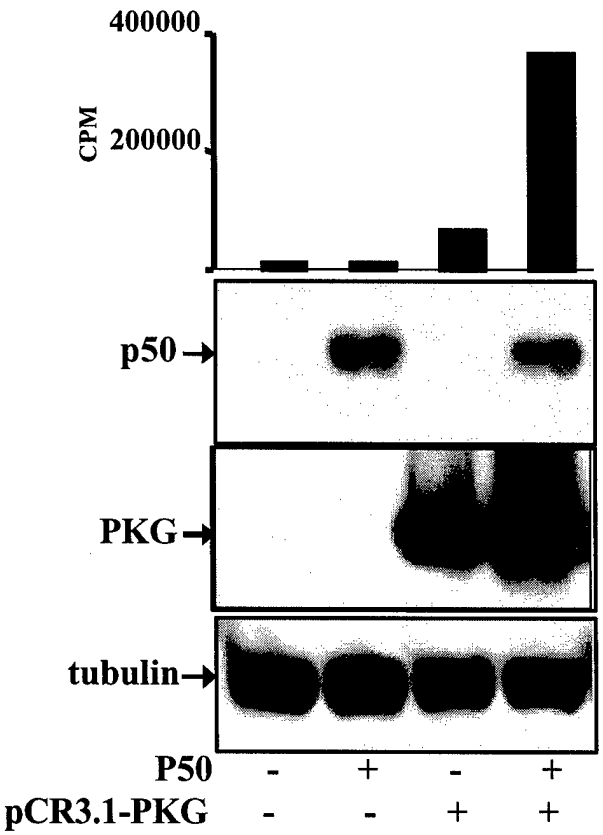
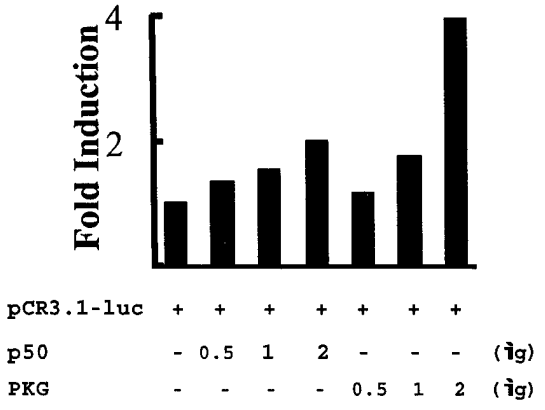
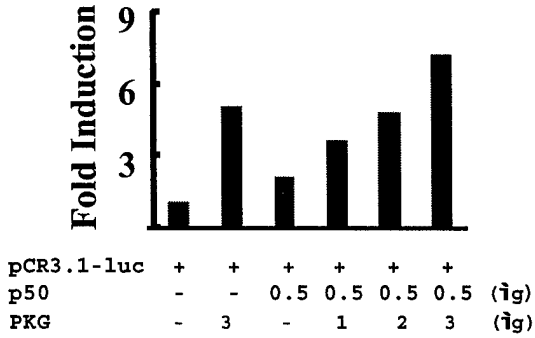
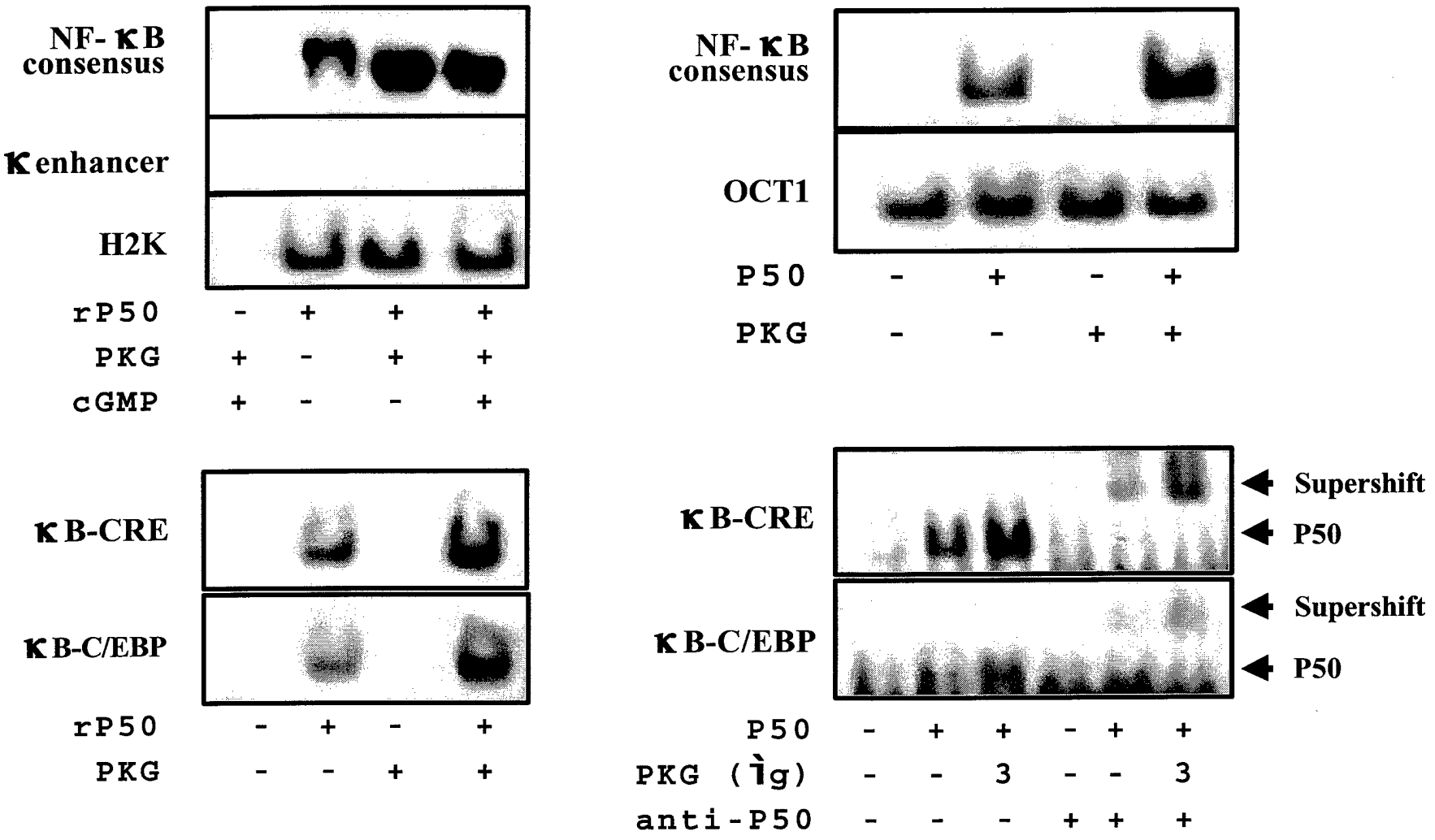
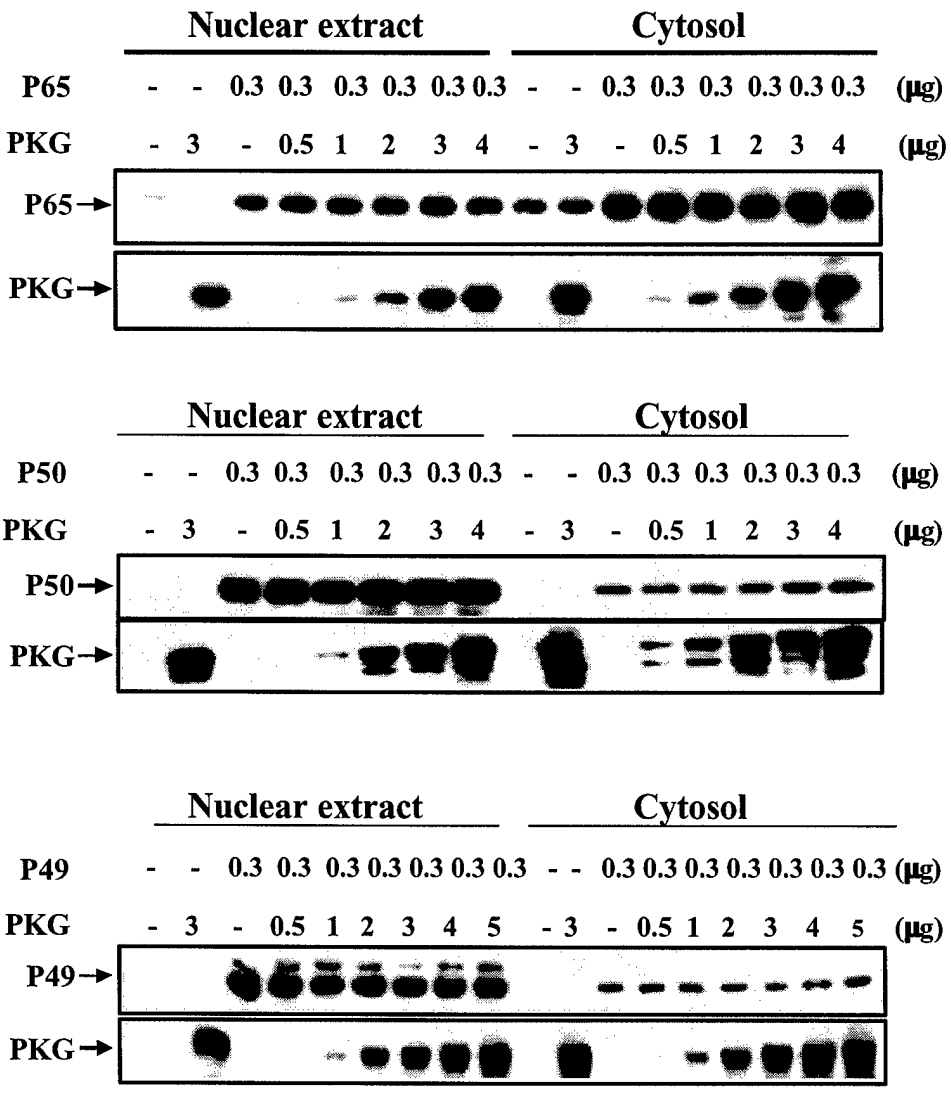


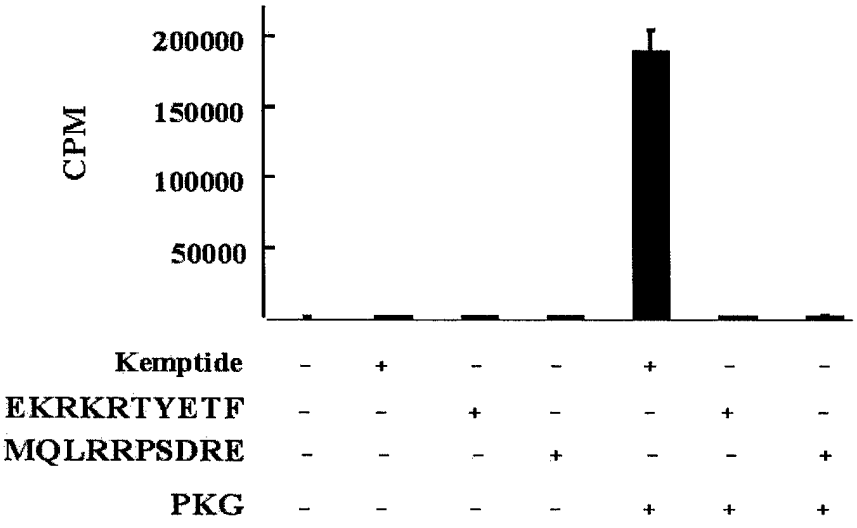




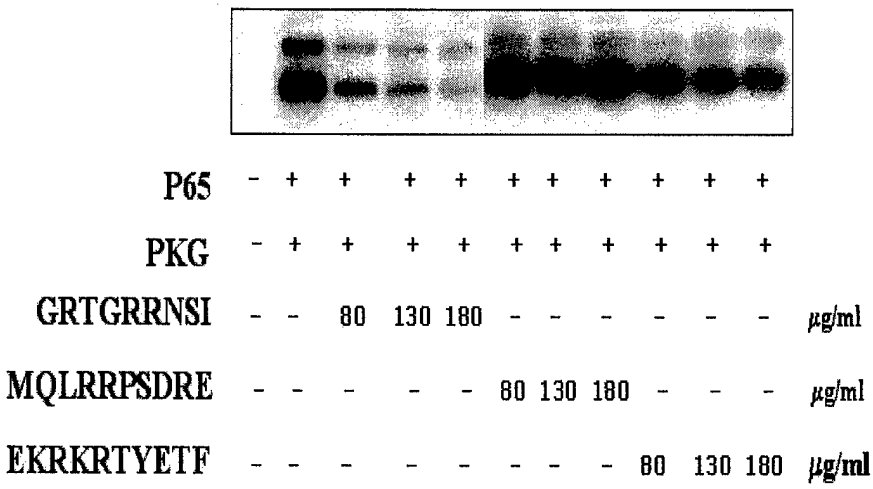

NF-κB consensus
κenhancer
H2K
rP50 - + + +
PKG + - + +
cGMP + - - +
κB-CRE
κB-C/EBP
rP50 - + - +
PKG - - + +
NF-κB consensus
OCT1
P50 - + - +
PKG - - + +
κB-CRE
κB-C/EBP
Supershift
P50
Supershift
P50
P50 - + + - + +
PKG (μg) - - 3 - - 3
anti-P50 - - - + + +


Nuclear extract
Cytosol
P65 - - 0.3 0.3 0.3 0.3 0.3 0.3 - - 0.3 0.3 0.3 0.3 0.3 0.3 (μg)
PKG - 3 - 0.5 1 2 3 4 - 3 - 0.5 1 2 3 4 (μg)
P65
PKG
Nuclear extract
Cytosol
P50 - - 0.3 0.3 0.3 0.3 0.3 0.3 - - 0.3 0.3 0.3 0.3 0.3 0.3 (μg)
PKG - 3 - 0.5 1 2 3 4 - 3 - 0.5 1 2 3 4 (μg)
P50
PKG
Nuclear extract
Cytosol
P49 - - 0.3 0.3 0.3 0.3 0.3 0.3 0.3 - - 0.3 0.3 0.3 0.3 0.3 0.3 0.3 (μg)
PKG - 3 - 0.5 1 2 3 4 5 - 3 - 0.5 1 2 3 4 5 (μg)
P49
PKG

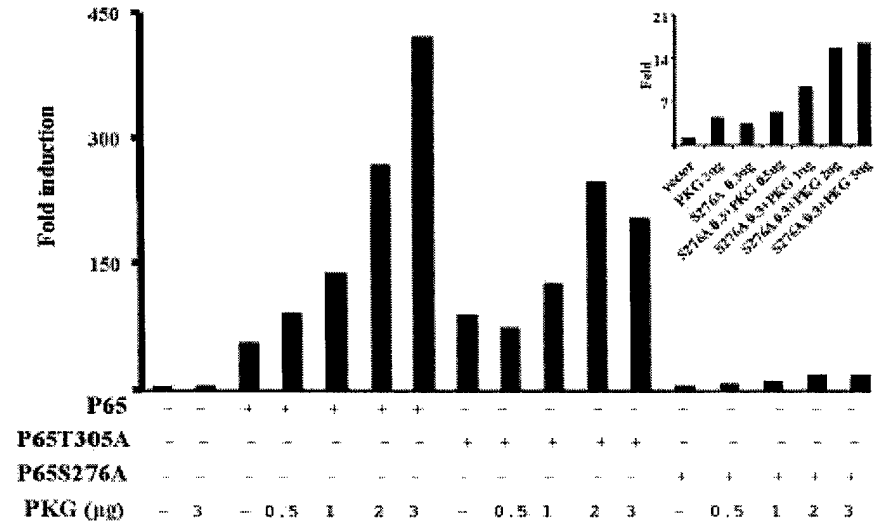
A


CPM
200000
150000
100000
50000
Kemptide – + – – + – –
EKRKRTYETF – – + – – + –
MQLRRPSDRE – – – + – – +
PKG – – – – + + +

B


P65 – + + + + + + + + + +
PKG – + + + + + + + + + +
GRTGRRNSI – – 80 130 180 – – – – – – μg/ml
MQLRRPSDRE – – – – – 80 130 180 – – – μg/ml
EKRKRTYETF – – – – – – – – 80 130 180 μg/ml

C


Fold induction
450
300
150
P65 – – + + + + + – – – – – – – – – – –
P65T305A – – – – – – – + + + + + – – – – – –
P65S276A – – – – – – – – – – – – + + + + +
PKG (μg) – 3 – 0.5 1 2 3 – 0.5 1 2 3 – 0.5 1 2 3

LAHIVE & COCKFIELD LLP

COUNSELLORS AT LAW
28 STATE STREET
BOSTON, MASSACHUSETTS 02109-1784
TELEPHONE (617) 227-7400
FAX (617) 742-4214
lc@lahcoc.com

17 April 2000

Assistant Commissioner for Patents
Box PCT
Washington, D.C. 20231

Re: PCT Application Entitled **METHODS AND COMPOSITIONS FOR MODULATING AN IMMUNE RESPONSE**
Applicant: **DANA-FARBER CANCER INSTITUTE, INC. et al.**
Filed: **Herewith**
Our File: **CMZ-121CPPC**

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Signature

Debra J. Milasincic
Please Print Name of Person Signing

Respectfully submitted,

Catherine J. Kara
Reg. No. 41,106
Attorney for Applicant

Enclosures

area of infection and succumb to systemic bacteremia (Patarca *et al.* (1990) *Immunol. Rev.* (116:1-16). Eta-1/osteopontin expression has also been linked to granuloma formation, where it may regulate the chronic cellular response associated with tuberculosis infection and silicosis (Nau *et al.* (1997) *Proc. Natl. Acad. Sci. USA* 94:6414-6419. Moreover, in experimental glomerulonephritis, neutralizing antibodies to osteopontin greatly diminish the influx of macrophages and T-cells and reduce kidney damage (Yu *et al.* (1998) *Proc. Am. Assoc. Physicians* 110:50-64). While Eta-1/ostepontin has been implicated in at least certain immunological reactions, its precise role in the immune system has not previously been established. Moreover, Eta-1 is a multifunctional protein having diverse biological roles including , but not limited to, bone resorption, neoplastic transformation, atheromatous plaque formation, dystrophic calcification of inflamed and/or damaged tissues and resistance to certain bacterial infections. (See *e.g.*, Oldberg *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:8819; Ross *et al.* (1993) *J. Biol. Chem.* 268:9901; Giachelli *et al.* (1995) *Ann. NY Acad. Sci.* 760:109; Senger *et al.* (1983) *Nature* 302:714; and Srivatsa *at al.* (1997) *J. Clin. Invest.* 99:996). With regards in particular, to understanding the role of Eta-1/osteopontin in immunity, there exists a need to understand the precise role that Eta-1/osteopontin plays in regulating immune responses in order to develop new approaches to treating immune disorders and diseases.

Summary of the Invention

The present invention establishes that Eta-1/osteopontin is a critical regulator of type-1 (cell-mediated) immunity and that this molecule includes a domain that promotes the production of the type 1 cytokine IL-12 and a domain that inhibits the production of the type 2 cytokine IL-10. Thus, the invention provides for the use of Eta-1/osteopontin modulatory agents (i.e, agents that stimulate or inhibit Eta-1/osteopontin activity) to bias an immune response either toward type 1 or type 2 immunity, depending on the clinical situation. The present invention identifies Eta-1/osteopontin as a critical cytokine in type 1 immune responses, in particular, in delayed type hypersensitivity responses. The invention defines Eta-1/osteopontin as a multifunctional molecule which acts as both a stimulator of IL-12 secretion by macrophages and an inhibitor of IL-10 expression. As such, Eta-1/osteopontin serves to bias an organism's cytokine pattern

towards that of a type 1 immune response. In particular, induction of IL-12 and inhibition of IL-10 reflect differential engagement of macrophage receptors: a phosphorylation-dependent interaction between the N-terminal portion of Eta-1/osteopontin and its integrin receptor on macrophages leads to IL-12 expression, while a phosphorylation-independent interaction of a C-terminal domain of Eta-1/osteopontin with CD44 mediates IL-10 inhibition. Moreover, cleavage of Eta-1/osteopontin by thrombin results in a C-terminal fragment of the cytokine which interacts with CD44 and induces macrophage chemotaxis, while engagement of integrin receptors by a non-overlapping N-terminal fragment leads to macrophage spreading and activation.

Based, at least in part, on a detailed understanding of the role this multifunctional cytokine plays in type 1 immune responses, the present invention features novel approaches to modulating immune responses, in particular, in potentiating type 1 immune responses. The invention further features new methods of treating disorders that may benefit from either a type 1 or type 2 immune response. More specifically, the identification of Eta-1/osteopontin as a critical regulator of type 1 immunity allows for selective manipulation of T cell subsets in a variety of clinical situations using the modulatory methods of the invention. The stimulatory methods of the invention (*i.e.*, methods that use an Eta-1/osteopontin stimulatory agent) upregulate the production of the Th1-associated cytokine IL-12 and/or dowregulate the production of the Th2-associated cytokine IL-10, with concomitant promotion of a type 1 immune responses and downregulation of type 2 immune responses. These stimulatory methods that promote a type 1 response can be used, for example, in the treatment of infections (e.g., bacterial, viral), cancer, allergy, burn-associated sepsis and immunodeficiency disorders. In contrast, the inhibitory methods of the invention (*i.e.*, methods that use an Eta-1/osteopontin inhibitory agent) downregulate the production of the Th1-associated cytokine IL-12 and/or upregulate the production of the Th2-associated cytokine IL-10, with concomitant downregulation of a type 1 immune responses and promotion of type 2 immune responses. These inhibitory methods that promote type 2 responses can be used, for example, in the treatment of autoimmune disorders, transplant rejection, granulomatous disorders, herpes simplex keratitis and bacterial arthritis.

Moreover, based on a detailed understanding of the functional domains of Eta-1/osteopontin, the present invention provides biosynthetic molecules which mimic distinct functions of Eta-1/osteopontin for use in a variety of therapeutic applications, in particular, in wound healing, enhancement of the immune response and in treatment of granulomatous disease. In particular, the biosynthetic molecules of the present invention are useful in biasing an immune response towards a delayed type hypersensitivity response, *i.e.*, towards type 1 immunity. A preferred IL-12 stimulatory domain of Eta-1/osteopontin comprises amino acids 71-168 of SEQ ID NO: 2. A preferred IL-10 inhibitory domain of Eta-1/osteopontin comprises amino acids 169-266 of SEQ ID NO: 2. Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawings

Figure 1A-C demonstrates gramuloma formation in normal, cytokine-deficient and Eta-1/osteopontin-deficient mice. *Figure 1A* depicts the data as the mean number of granulomas per high-power field (HPF) (X200 magnification), mean number of cells per granuloma, and as the product of these two indices, termed "granuloma burden". (Error bars indicate 1 SEM.). *Figure 1B* depicts an analysis of surface antigens expressed by cells within granulomas in the indicated mouse strains. *Figure 1C* depicts cytokine expression by cells from lymph nodes draining the site of granulomas. Data are representative of three independent experiments.

Figure 2A-E demonstrates HSV-1-specific delayed-type hypersensitivity (DHT) reactions in normal and Eta-1/opn$^{-/-}$ mice. *Figure 2A* depicts footpad swelling in Eta-1/opn$^{-/-}$ vs. Eta-1/opn$^{+/+}$ mice inoculated with HSV-1. The right (control, □) and left (HSV-1, ■) footpads of each mouse were measured 24h later using a micrometer. Each data point represents the mean and standard error of three mice/group. *Figure 2B* depicts inhibition of the HSV-1 DHT response in Eta-1/opn$^{+/+}$ mice by acute depletion of Eta-1/opn. *Figure 2C* depicts HSK in Eta-1/opn$^{-/-}$ (open circles) vs. Eta-1/opn$^{+/+}$ (closed circles) mice inoculated with HSV-1. *Figure 2D* depicts HSK in BALB/cBγJ (open circles), Eta-1/opn$^{-/-}$ (closed circles), Eta-1/opn$^{+/+}$ (open squares), and CB-17 (closes squares) mice inoculated with HSV-1. *Figure2E* depicts the cytokine response after